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PRODUCTION OF IMMUNITY AGAINST EXPERIMENTAL *BR. MELITENSIS* INFECTION IN GOATS

A Comparison of Two Killed and One Living Vaccine

By

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INTRODUCTION

This experiment forms part of a series, sponsored by the joint FAO/WHO Expert Committee on Brucellosis, to evaluate some of the available methods of immunization of goats against *Br. melitensis* infection. Renoux, Alton and Amarasinghe (1957) demonstrated protection against *Br. melitensis* infection in goats by the use of smooth killed *Br. melitensis* in adjuvant. Smooth cultures in adjuvant produce, however, a marked and prolonged agglutinin response which is undesirable in an eradication programme. An adjuvant vaccine prepared from a killed rough culture of *Br. melitensis* produced the same degree of immunity in guinea pigs, without producing serum agglutinins, as a similar vaccine prepared from a smooth strain (Jones and Alton, 1956). Elberg and Faunce (1957) obtained good protection in pregnant goats by using a living strain of *Br. melitensis* reported to be smooth and avirulent. This strain is a "non-dependent" mutant which was isolated from a "streptomycin-dependent" population.

Initially the intention was to compare the value of the rough and smooth adjuvant vaccines in goats, but later the living vaccine developed by Elberg became available and was included. Three groups of goats were vaccinated, group 1 with the killed rough adjuvant vaccine, group 2 with the killed smooth adjuvant vaccine and two months later, group 3 with living vaccine of Elberg. Four months after vaccinating the goats in group 3, all vaccinated and control goats were challenged with graded doses of a virulent strain of *Br. melitensis* and the ID_{50} of each group was assessed by autopsy and bacteriological examination of tissues four weeks after challenge.

MATERIALS AND METHODS

Preparation of vaccines. The rough variant, designated as R6 in the previous experiment (Jones and Alton, 1956) had been obtained from a broth culture of *Br. melitensis* strain H38 and preserved by drying. For vaccine production it was grown for seventy-two hours on Roux flasks of Albimi agar. The smooth culture of H38, which had been dried for use as a challenge strain, was similarly reconstituted and grown on Roux flasks of serum dextrose agar rather than Albimi agar since the latter

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favoured dissociation. Viable counts of the bacterial suspensions were carried out before killing by heat at 70°C for one hour in a water bath. In the preparation of the vaccine 160 ml. of light liquid paraffin and 40 ml. of Falba were emulsified for five minutes in an electric mixer with 50 ml. of bacterial suspension containing 250×10^9 heat-killed organisms per ml. The resulting vaccine was sterile and the emulsion was stable when inoculated into goats three days after its preparation. Each goat received a dose of 10^{11} organisms contained in two ml. of the vaccine.

The living vaccine was prepared in Elberg's laboratory and dispatched by air from California. The viable count performed on the day it was received and inoculated into goats was 2×10^9 organisms per ml. Each goat was inoculated with one ml. of the vaccine.

Animals and housing. Non-pregnant female goats from one to eight years of age were obtained from commercial sources in England. The goats were bled and the sera were tested for the presence of brucella agglutinins and complement-fixing antibodies on at least two occasions prior to vaccination. Four goats which showed a partial reaction at 1/20 in the agglutination test were excluded from the experiment. Brucella were not recovered from the tissues of these goats at autopsy. Before challenge each vaccine group and the control group was divided into three sub-groups and kept in separate isolation units.

Vaccination of goats. On 3rd September, 1956 thirty-three goats were inoculated subcutaneously behind the shoulder, each with two ml. of the rough adjuvant vaccine which was dispersed by rubbing the area after inoculation. A further thirty-three animals were inoculated with two ml. of the smooth adjuvant vaccine. Forty-eight hours after vaccination some of the goats had a slight swelling and heat at the site of inoculation. Seven days after vaccination a swelling was apparent in nearly all the goats: in a number it seemed to be painful, but it receded and in no case did an abscess appear. At autopsy seven months later caseous nodules and fibrosis were observed at the site of inoculation in nearly all the goats.

On 29th October 1956, thirty-four goats were inoculated with one ml. of the living vaccine of Elberg. Ten vaccinated goats died or were killed before challenge, the most frequent cause being injury. All were negative to brucella upon bacteriological examination of tissues at autopsy.

Challenge infection. The challenge inoculum was obtained from a dried culture of *Br. melitensis* strain H38 which was used as a challenge strain in previous vaccination experiments in this series. The culture produced smooth colonies which in their serology, H_2S production and growth on dyes were typical of *Br. melitensis*. Forty-eight hour growth on serum-dextrose agar was suspended in peptone water. The goats were challenged on 18th February, 1957, by placing on the conjunctiva of the right eye one drop of the appropriate dilution from a standardized pipette, to give doses of 10^4, 10^5 and 10^6 organisms per goat in the sub-groups of the controls, and 10^6, 10^7 and 10^8 organisms per goat in the vaccinated sub-groups.

Autopsy and examination of tissues. Beginning on 18th March, 1957, one goat from each sub-group (i.e. twelve goats) was autopsied per day from the twenty-eighth to the thirty-ninth day after challenge. From each goat twenty-four tissues were removed and cultured on Albimi agar containing antibiotics. The plates were examined after four and eight days incuba-

tion and the numbers of colonies per plate estimated. A maximum of culture, typing and ID_{50} determination have been reported previously (Jones and Alton, 1956).

Serological studies. Vaccinated goats were bled twice a week for two weeks, at weekly intervals for the next eight weeks and fortnightly thereafter. Control goats were done monthly. All goats were bled ten and twenty-one days after challenge and at autopsy.

All sera were tested by the tube agglutination method using a smooth melitensis antigen which had been standardized to give fifty per cent agglutination with 1/480 dilution of the International Standard Anti-*Brucella abortus* Serum. The tests were read after twenty-four hours incubation at 37°C. The titres of the sera have been expressed as the final dilution which caused fifty per cent agglutination. In terms of International Units 1/40 is equivalent to 83 units, 1/80 to 166 units etc. (Stableforth, 1954). Sera obtained at autopsy were also tested using 5 per cent saline as diluent in the tube agglutination test as it had been reported by many workers to give more positive results with goat and sheep sera than the usual method.

Most sera were examined for complement-fixing antibodies using a trichloracetic acid extract of *Br. melitensis* as an antigen. The gel-diffusion precipitin plate test (Bruce and Jones, 1958) was employed by using the trichloracetic acid extract of *Br. melitensis* as antigen with a representative number of sera.

RESULTS

The number of infected goats in each vaccine and challenge group is given in Table 1 and plotted in Graph 1. The recovery of a single colony of the challenge strain of *Br. melitensis* was considered to be evidence of infection of the animal. The ID_{50} for each vaccine was significantly different from that of the controls. The number of organisms required to infect fifty per cent of the goats vaccinated with the living vaccine is approximately sixty-two times that required to infect fifty per cent of the controls. The ID_{50} for the smooth vaccine was fifty times that of the controls, that of the rough vaccine was ten times that of controls. The latter seemed to afford the least protection, although the differences among the vaccines were not statistically significant as evidenced by the overlapping of the confidence limits.

This system of evaluating the results of challenge infection does not take any account of the degree of infection discovered in different goats on autopsy. Tables 2 to 5 give the approximate numbers of brucella colonies recovered from each tissue of every goat. It can be seen that in many goats, especially in the living vaccine group, only very scanty infection of the regional lymph nodes draining the eye could be demonstrated. In many of the goats inoculated with killed vaccines, however, the infection was not restricted to the regional lymph nodes.

A goat was considered to have a generalized infection if one or more *Br. melitensis* colonies were recovered from any tissue other than the atlantal, parotid, retropharyngeal and submaxillary lymph

nodes. Twelve of 29 goats in the rough adjuvant vaccine group, nine of 29 in the smooth adjuvant group and one of 32 goats in the living vaccine group had generalized infection. The significance of the differences between the generalized infection in the living vaccine group and those in the two adjuvant vaccine groups, was tested as follows: in the analysis of the 2-way table of proportions, constants for the rows and columns were fitted by iteration to a maxiumum likelihood solution (Table 7). The effects shown in the second column of the table are differences between the constants for the three vaccines fitted to the data in Table 6. The mathematical basis of this method has been outlined by Yates (1955), the method has been developed by F. B. Leech and the computations carried out on the "401" electronic computer at Rothamsted Experimental Station. There was no difference between the generalized infections in the rough and smooth adjuvant vaccine groups.

In Table 6 the ID_{50}s, determined on the basis of generalized infection, are given in conjunction with the results obtained by the original interpretation. When the ID_{50} of the controls is compared with those of the vaccinated groups it can be seen that the killed vaccines gave approximately the same degree of protection, whereas the living vaccine gave significantly greater protection against generalized infection than was apparent in the original comparison.

TABLE 1

DETERMINATION OF THE ID_{50} IN VACCINATED AND CONTROL GOATS

Vaccine	*Challenge dose*	*Numbers infected* inoculated*	*Infection*		ID_{50}	*Confidence limits*	*Chi² of the ** line*
			Percent observed	*Percent expected*			
Controls	10^4	1/10	10·0	4·5	4×10^5	$1\cdot3\times10^5$ to $1\cdot2\times10^6$	1·01
	10^5	2/10	20·0	20·0			
	10^6	7/11	63·7	71·4			
Rough adjuvant	10^6	1/9	11·1	9·1	4×10^6	$1\cdot3\times10^6$ to $1\cdot2\times10^7$	0·06
	10^7	8/10	80·0	81·8			
	10^8	10/10	100·0	100·0			
Smooth adjuvant	10^6	1/9	11·1	6·3	2×10^7	$3\cdot8\times10^6$ to 1×10^8	0·53
	10^7	3/10	30·0	36·4			
	10^8	10/10	100·0	100·0			
Living	10^6	2/10	20·0	9·1	$2\cdot5\times10^7$	$6\cdot6\times10^6$ to $9\cdot4\times10^7$	2·25
	10^7	3/11	27·3	29·4			
	10^8	7/11	63·7	75·0			

* Recovery of one or more colonies of the challenge strain from any tissue.

** In each of the groups the number of degrees of freedom is one and to indicate good fit the Chi² of the line must be less than 3·8.

Persistence of the living vaccine strain in tissues of goats. Two goats which died fourteen and fifteen weeks after inoculation of the living vaccine were negative to brucella upon bacteriological examination

of tissues. The vaccine strain was recovered in very small numbers from the left suprascapular lymph node of one goat at autopsy twenty weeks after vaccination. From another goat in this group the vaccine strain was isolated from the iliac and supramammary lymph nodes and the challenge strain was recovered from the right submaxilliary lymph node. The vaccine strain was distinguished from the infecting strain by its greater senisitivity to basic fuchsin and thionin.

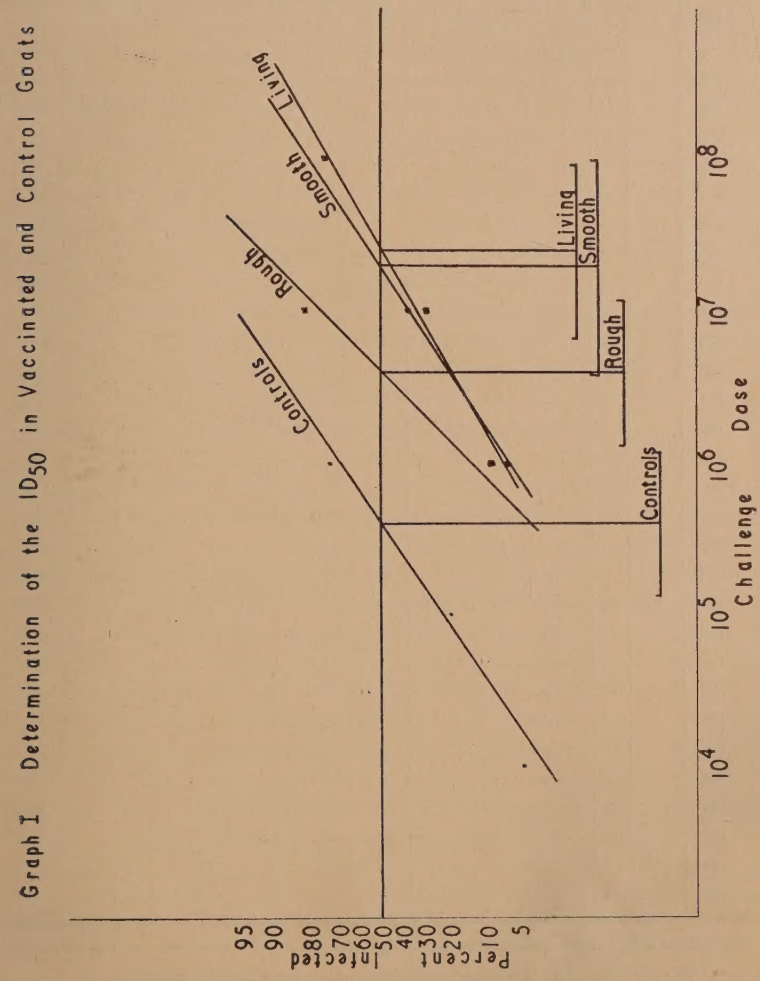

TABLE 2

DEGREE OF *Br. melitensis* INFECTION IN TISSUES OF NON-VACCINATED GOATS

Goat number	*Challenge dose*	*Type of infection*	*Atlantal*	*R. parotid*	*L. parotid*	*R. retropharyngeal*	*L. retropharyngeal*	*R. submaxillary*	*L. submaxillary*	*Hepatic*	*Iliac*	*R. supra-mammary*	*L. supra-mammary*	*Mediastinal*	*Mesenteric*	*R. precrural*	*L. precrural*	*R. suprascapular*	*L. suprascapular*	*Kidney*	*Liver*	*Lung*	*Mammary gland*	*Spleen*	*Uterus*
			Localized infection							*Generalized infection*															
193	10^4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
194		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
198		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
201		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
202		L	—	2	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
203		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
204		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
208		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
209		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
210		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
186	10^5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
188		L	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
190		G	3	4	2	3	3	4	3	2	2	2	2	2	—	2	2	3	3	—	—	1	—	3	—
191		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
196		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
206		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
200		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
205		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
199		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
207		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
189*	10^6	G	—	2	—	3	2	4	—	—	—	—	—	—	—	—	—	2	1	—	—	—	—	—	—
147		G	3	2	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—
150		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
166		G	4	4	—	1	—	2	1	—	2	1	—	—	—	—	—	1	2	—	—	—	—	2	—
177		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
183		G	2	3	2	2	2	2	2	1	—	2	2	2	—	2	2	2	2	—	—	—	—	1	—
184		G	2	3	1	2	2	2	2	1	1	2	2	1	1	1	1	1	2	—	—	—	1	1	—
185		G	2	4	3	2	2	2	3	—	—	—	1	2	—	—	2	1	2	—	—	—	—	2	—
187		L	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
197		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
164		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

*Died 9 days before autopsies were due to begin.

The estimated number of colonies recovered from each tissue is recorded as follows:
1 = less than 10 colonies, 2 = 10 to 100 colonies, 3 = 100 to 1,000 colonies, 4 = more than 1,000 colonies. L = Localized Infection only. G = Generalized Infection.

TABLE 3

DEGREE OF *Br. melitensis* INFECTION IN TISSUES OF GOATS INOCULATED WITH ROUGH ADJUVANT VACCINE

Goat number	Challenge dose	Type of infection	Localized infection							Generalized infection														
			Atlantal	R. parotid	L. parotid	R. retropharyngeal	L. retropharyngeal	R. submaxillary	L. submaxillary	Hepatic	Iliac	R. supra-mammary	L. supra-mammary	Mediastinal	Mesenteric	R. precrural	L. precrural	R. suprascapular	L. suprascapular	Kidney	Liver	Lung	Mammary gland	Spleen	Uterus
68	10^6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
78		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
90		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
94		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
97		G	2	3	2	3	2	3	—	2	—	1	—	2	1	1	1	2	2	—	—	—	2	—
107		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
108		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
126		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
133		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
72	10^7	G	2	3	2	3	3	2	2	—	3	2	2	2	—	1	1	2	—	—	—	—	2	—
73		L	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
75		G	2	3	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	2	—
85		L	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
91		L	1	—	—	1	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
96		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
99		L	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2043		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2072		L	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2032		L	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
82	10^8	G	4	4	2	4	4	3	3	—	2	2	3	4	1	1	2	2	2	—	1	—	3	—
84		G	3	4	2	1	—	3	2	—	2	2	1	2	1	1	2	2	2	1	1	1	3	—
86		G	3	4	2	2	2	2	—	1	2	2	1	1	1	1	2	—	2	—	—	—	2	—
87		G	4	4	3	3	3	3	3	2	3	3	3	3	1	3	3	3	3	2	1	—	3	3	—
102		G	2	4	4	4	4	2	4	2	1	2	2	2	—	2	3	2	2	—	1	—	—	3	—
110		L	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
116		G	2	3	3	2	2	3	3	2	1	2	1	3	—	3	2	3	2	—	—	—	2	—
117		G	2	3	2	2	3	2	2	—	1	2	1	2	1	1	2	2	2	—	—	—	2	—
2055		G	3	3	—	1	1	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	1	—
2073		G	2	3	2	4	4	1	—	1	—	—	—	2	—	2	3	1	4	—	—	1	—	2	—

TABLE 4

DEGREE OF *Br. melitensis* INFECTION IN TISSUES OF GOATS INOCULATED WITH SMOOTH ADJUVANT VACCINE

Goat number	Challenge dose	Type of infection	Localized infection							Generalized infection															
			Atlantal	R. parotid	L. parotid	R. retropharyngeal	L. retropharyngeal	R. submaxillary	L. submaxillary	Hepatic	Iliac	R. supra-mammary	L. supra-mammary	Mediastinal	Mesenteric	R. precrural	L. precrural	R. suprascapular	L. suprascapular	Kidney	Liver	Lung	Mammary gland	Spleen	Uterus
104	10^6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
121		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
69		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
76		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
81		G	—	1	1	—	—	1	1	—	—	1	—	1	—	—	—	—	—	—	—	—	—	—	—
122		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
132		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2031		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2042		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
77	10^7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
80		L	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
83		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
89		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
101		L	—	1	—	2	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
134		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
137		G	1	4	3	3	2	3	3	2	1	2	2	2	—	1	—	2	2	2	2	—	—	3	—
139		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
140		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2054		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
74	10^8	G	3	4	—	1	—	3	1	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
93		G	3	4	2	2	1	2	2	—	1	1	2	—	—	1	2	1	1	—	—	—	2	1	—
98		G	—	2	—	—	1	1	1	—	—	—	2	—	—	—	—	1	—	—	—	—	—	—	—
106		L	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
113		L	—	2	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
114		G	2	2	—	2	2	2	—	—	2	—	—	—	—	—	3	—	—	—	—	—	—	—	—
119		L	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
120		G	1	3	2	2	—	1	2	1	1	1	1	—	—	1	2	1	—	—	—	—	1	2	—
2057		G	2	3	1	2	1	2	2	—	—	1	1	—	—	—	1	1	2	—	—	—	—	1	—
2070		G	1	3	1	2	3	3	3	1	—	—	1	3	—	2	2	2	1	—	—	—	—	2	—

TABLE 5

DEGREE OF *Br. melitensis* INFECTION IN TISSUES OF GOATS INOCULATED WITH LIVING VACCINE

Goat number	Challenge dose	Type of infection	Localized infection							Generalized infection													
			Atlantal	R. parotid	L. parotid	R. retropharyngeal	L. retropharyngeal	R. submaxillary	L. submaxillary	Hepatic	Iliac	R. supra-mammary	L. supra-mammary	Mediastinal	Mesenteric	R. precrural	L. precrural	R. suprascapular	L. suprascapular	Kidney	Liver	Lung	Mammary gland	Spleen	Uterus
142	10^6	—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
136		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
131		L		–	–	–	–	1		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
144		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
152		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
160		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
161			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
'2056		L	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
'2060		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
'2076		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
'143	10^7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
153		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
166		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
157		L	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
158		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
159			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
162		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
163		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
171		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
175		L	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
'2066		L	–	–		–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
172	10^8	L	–	1	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
135		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
141		L	–	2	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
145		L	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
148		L	1	3	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
149		—	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
154		L	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
167		L	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
168		G	–	1	1	–	–	1	1	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–
169		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2053		—	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

TABLE 6

ANALYSIS OF VACCINATION EXPERIMENT IN TERMS OF GENERALIZED INFECTION

Vaccine group	*Challenge dose*	*Numbers of goats showing*		*ID_{50} On basis of:*	
		*Infection**	*Generalized infection†*	*Infection**	*Generalized infection†*
Controls	10^4	1/10	0/10		
	10^5	2/10	1/10	400,000	800,000
	10^6	7/11	6/11		
Rough adjuvant	10^6	1/9	1/9		
	10^7	8/10	2/10	4,000,000	18,000,000
	10^8	10/10	9/10	(10 x)‡	(22 x)
Smooth adjuvant	10^6	1/9	1/9		
	10^7	3/10	1/10	20,000,000	47,000,000
	10^8	10/10	7/10	(50 x)	(59 x)
Living	10^6	2/10	0/10		
	10^7	3/11	0/11	25,000,000	600,000,000§
	10^8	7/11	1/11	(62 x)	(750 x)

*Infection = One or more *Br. melitensis* colonies recovered from any tissue.

†Generalized infection = One or more *Br. melitensis* colonies recovered from any tissue other than the atlantal, parotid, retropharyngeal and submaxillary lymph nodes.

‡The figures in brackets denote the number of times the ID_{50} of the vaccinated group is greater than that of the controls.

§The ID_{50} for the living vaccine was estimated by plotting a single point on log probability paper and drawing the line parallel to that of the controls.

Serological Observations

Agglutination test. Goats vaccinated with the rough adjuvant vaccine showed no significant agglutination response. Only one had a titre of more than 1/10—a temporary reaction at 1/40. The smooth adjuvant vaccine, as expected, caused a marked and prolonged agglutination response, to a maximum of between 1/80 and 1/640. Six goats still had a titre of 1/320 six months after vaccination. Goats vaccinated with the living vaccine showed a maximum agglutination response (1/40 to 1/640) between seven and fourteen days after vaccination. The titre of all goats receded gradually, but at four months they still ranged from 1/10 to 1/160.

The agglutinin reactions following challenge did not give a reliable indication of infection. Only six of the twelve goats showing generalized infection in the rough adjuvant group displayed a rise in agglutinin titre. Of the six goats in the control group which had a generalized infection two had a serum reaction of 1/40 or greater, two had a titre of 1/10 and two were negative. Of thirty-one sera from goats in the control and rough adjuvant vaccine groups which proved to be free of infection, five had agglutination titres from 1/10 to 1/20. The serum titres of goats which had been inoculated with

smooth adjuvant and living vaccines were not altered by the challenge infection.

The end-points obtained with five per cent saline in the agglutination test were not significantly different from those obtained in the ordinary test. Evidence of "blocking" antibodies was not detected in any of the sera.

TABLE 7

Vaccine	*Mean percent infected*	*Significance tests of difference between living and adjuvant vaccines*		
		Effect in logits (base 2) ± *S.E.*	*t*	*P*
Smooth ..	39·35%	2·464 ± ·871	2·83	·01
Living ..	6·85%			
Rough ..	37·12%	3·066 ± ·907	3·38	·001

Complement-fixation test. Complement-fixing antibodies were not detected before challenge in the sera of goats receiving the rough adjuvant vaccine with the exception of five goats with temporary reactions of not more than 1/20. The smooth adjuvant vaccine stimulated the production of complement-fixing antibodies in the majority of animals and these persisted throughout the six month period before challenge. In the Elberg vaccine group complement-fixing antibodies were demonstrated in the sera of nearly all goats three weeks after vaccination. They had returned to negative by fifteen weeks, except for nine goats which remained positive.

Some goats appeared to be incapable of producing complement-fixing antibodies in response to vaccination with smooth cultures, although all produced agglutinins and precipitins. The complement-fixation test was less effective than the agglutination test in detecting infected goats following challenge.

Plate precipitin test. Precipitin lines were never observed with sera from goats receiving the rough adjuvant vaccine. Sera from the smooth adjuvant group were positive in the precipitin test from the second week following vaccination to the time of challenge. All the goats in the Elberg vaccine group had serum precipitins at two weeks but they persisted in only twelve cases for the fifteen week period.

Sera obtained at autopsy from goats in the control and rough adjuvant groups gave the following results. Of eighteen sera from goats with a generalized infection eight had precipitins, whereas nine had agglutinins greater than 1/20, and six had agglutinins in the range from 1/10 to 1/20. Of thirty-one sera from goats shown to be free of infection five had agglutinins from 1/10 to 1/20 but none had precipitins.

DISCUSSION

The comparison of the efficacy of the rough and smooth adjuvant vaccines in goats confirmed the results previously obtained in guinea pigs. Significant protection was afforded by both vaccines. The difference between the rough and smooth vaccines, though greater than that obtained in guinea pig experiments, was still not significant. When the criterion of infection was the isolation of one or more colonies from a goat, the living vaccine did not stimulate significantly more protection than the killed vaccines. However, when generalization of infection in the animal was the criterion the living vaccine was the most effective agent.

Although the interval between vaccination and challenge was two months shorter for the living vaccine group it does not seem likely that the immunity would have diminished significantly in two additional months. The superiority of the living vaccine in preventing generalized infection in non-pregnant goats and its efficacy in pregnant goats as reported by Elberg and Faunce (1957) suggest that this vaccine will provide better immunity under field conditions than either of the dead vaccines. However, the use of a living strain which stimulates agglutinin production presents obvious disadvantages in an eradication programme.

In contrast to the results obtained by Elberg and Faunce (1957) whereby heat-killed cells failed to stimulate immunity, the present results demonstrate that heat-killed cells are capable of producing significant protection if combined with an adjuvant. Such immunity is apparently not dependent on the presence of demonstrable serum antibodies inasmuch as the rough adjuvant vaccine which failed to stimulate agglutinin production nevertheless afforded protection.

CONCLUSIONS

The efficacy of three vaccines, (1) a rough killed culture of *Br. melitensis* in adjuvant, (2) a smooth killed culture of *Br. melitensis* in adjuvant and (3) a living avirulent strain of *Br. melitensis*, in preventing infection in non-pregnant goats following challenge with graded doses of virulent *Br. melitensis* was determined by autopsy and bacteriological examination of tissues four weeks after infection. Although the differences in the ID_{50} of the three vaccines were not significant the living vaccine was most effective in preventing a generalized infection. This superiority of the living vaccine in preventing generalized infection in non-pregnant goats suggest that it will provide better protection under field conditions than either of the dead vaccines despite the disadvantages of stimulating the production of agglutinins.

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STUDIES ON *PASTEURELLA SEPTICA* (*PASTEURELLA MULTOCIDA*)

Observations on some Biophysical Characters

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INTRODUCTION

It has been the experience of all workers who have studied *Pasteurella septica*, (*P. multocida*) that this organism, apart from frequent dissociation (mutation), is unstable in its biochemical reactions, viability etc. In most cases the results obtained by different workers are at variance. The wide variation in biological behaviour, particularly in respect of individual strains, has made this organism one of the least understood amongst the bacterial parasites causing diseases in animals. The present study was undertaken to examine some of the important characters of *P. septica*, especially of the strains which occur in India.

METHODS

A highly virulent buffalo strain was used for the development of the selective medium. Its growth was tested on blood agar media containing aesculin, crystal violet and cobalt chloride. For fermentation tests 49 freshly isolated strains were employed (buffalo 12, cattle 8, sheep 8, goats 6, and fowls 15). In order to avoid experimental errors the only strains used were those which gave a constant result on three consecutive tests, using different batches of medium at intervals of one week. The standard culture medium was peptone broth with neutral red as indicator, incubated for at least 28 days. Substrates used were glucose, sucrose, maltose, lactose, mannite, galactose, xylose, arabinose, sorbite, salicin, glycerol and trehalose.

Viability of the organism was tested by keeping heart blood and bones under different temperature conditions. Heart blood was kept (i) as dried material prepared from a thick film inside a test tube, (ii) by drying in air thick smears on slides, (iii) on cotton wool swabs, (iv) in test tubes, plugged with paraffined cotton wool, and (v) in sealed pipettes. The swabs, the rolled tubes and the dried smears were kept at room temperature (15 to 21°C). The blood tubes and the sealed pipettes were kept at room temperature, as well as at 37°C and cold room temperature (±4°C). Bones from haemorrhagic septicaemia cases were kept at room temperature as well as at 37°C. All specimens were examined after various intervals, culturally and biologically in mice.

For passaging through carbon dioxide two subcultures of a highly virulent buffalo strain were started. One series was grown on plain and the other on blood agar. The percentage of carbon dioxide was 25 per cent in the 1st to the 5th subculture; 35 per cent in 6th to 10th; 50 per cent in 11th to 15th; 60 per cent in 16th to 30th; 70 per cent in 31st to

70th and 100 per cent in the 71st to 146th subcultures. The abbreviations are C1, C2, C3 etc. for each subculture. The plain agar growth was discontinued after 11 passages. The blood agar line was continued up to the 146th subculture. The cultures were examined at every stage for any change in colony character and morphology, and at various intervals were tested for virulence in mice.

For testing antagonism of some of the common contaminating bacteria, a broth culture of *P. septica* mixed with heart blood from a carcass affected with pasteurellosis was mixed with *B. subtilis, B. anthracis, Staphylococcus, E. coli, Strept. pyogenes, Pseudomonas* and *C. pyogenes* respectively. The mixtures were incubated at 37°C with a control tube containing pure *P. septica* in the blood.

RESULTS

A Selective and Differential Culture Medium

It was observed that *P. septica* grows well on an agar medium containing 1:50,000 crystal violet and 1:1,000 aesculin and withstands cobalt chloride up to a maximum of about 150 p.p.m. At this strength cobalt chloride checked but did not completely inhibit the spreading of Proteus. To prepare the test medium the following constituents were used. Stock nutrient agar, 1,000 ml.; Crystal Violet (0·1 per cent), 2 ml.; Cobalt Chloride (10 per cent), 6 ml.; Aesculin, 1·0 g.

In this medium *P. septica* grows fairly well after 24 hours. The presence of crystal violet inhibits most staphylococci, *B. subtilis,* and many other airborne bacteria (Churchman, 1912; Edwards, 1931). Aesculin helps in identifying some dye-resistant contaminants, such as *Bact. coli, Strept. lactis* and some Staphylococci (Edwards, 1933). When there is contamination with a spreading type of Proteus, the colonies after 24 hours incubation are a pin point to a pin head in size. Although the very small ones may look like colonies of Pasteurella, on a careful examination, especially under a magnifying glass, a narrow thin film with a rough surface is found encircling the colonies of Proteus. These films gradually spread and at about 3 days the colonies attain a diameter of one half to one inch, particularly where a heavy inoculum has been used. As Pasteurella colonies were transferred to fresh medium within 48 hours the occasional extension of Proteus colonies did not prevent the successful isolation of *P. septica* in pure culture.

Sugar Fermentations

The percentages of strains fermenting the various substrates were as follows: Glucose—100; Sucrose—100; Galactose—90; Xylose—67; Mannite—32; Glycerol—22; Maltose—20; Sorbite—14; Arabinose—12; Trehalose—4; Lactose—0 and Salicin—0.

There was no significant difference in fermentation amongst the 49 strains which were collected from cattle, buffalo, sheep, goats and fowls, nor was there any correlation between sugar fermentation and virulence of the strains, all of which were tested for pathogenicity in

mice using a 24 hour broth culture in serial dilutions ranging from 10^{-3} to 10^{-9}. It was, however, interesting to note that amongst the 12 buffalo strains, 5, which were comparatively more virulent, fermented sorbite whereas the less virulent ones did not. On the other hand, the more virulent bovine strains did not ferment sorbite whereas the less virulent ones did (Table 1).

TABLE 1

MOUSE PATHOGENICITY OF BUFFALO AND BOVINE STRAINS

Source	*Strain No.*	*Dilution of culture*							*Fermentation of Sorbite*
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
Buffalo ..	HS 5	5*	5	5	0	0	0	0	—
	HS 6	5	5	5	0	0	0	0	—
	HS 7	5	5	5	2	0	0	0	—
	HS 8	5	5	4	3	0	0	0	—
	HS 14	5	5	5	3	0	0	0	—
	HS 22	5	5	2	0	0	0	0	—
	HS 4	5	5	4	1	0	0	0	—
	HS 9	5	5	5	5	5	0	0	+
	HS 10	5	5	5	5	5	2	0	+
	HS 19	5	5	5	5	5	5	0	+
	PBH	5	5	5	5	5	5	0	+
	P 129	5	5	5	5	5	5	0	+
Bovine ..	VI	5	5	5	5	5	0	0	—
	P 120	5	5	5	5	5	0	0	—
	P 52	5	5	5	5	5	5	0	—
	PB 3	5	5	5	5	5	5	5	—
	P 122	5	5	5	2	0	0	0	—
	P 124	5	5	5	0	0	0	0	—
	P 125	5	5	4	2	0	0	0	-
	P 126	5	5	3	3	0	0	0	-

*No. of mice dead out of 5.

Viability of P. septica

At room temperature, bone marrow was positive, both culturally and biologically, on the 13th day but not on the 16th day; at 37°C it was positive after 96 but not after 120 hours. On the 5th day, however, the marrow substance was very dry. It is quite likely that the disappearance of the organism so soon was due to the absence of moisture. According to Minett (1945) numerous organisms can be found in the marrow at room temperature after 8 days.

At room temperature the swabs of blood were positive after 118 but not after 166 hours, at which time they were completely dry. The thick smears on slides as well as those on roll-tubes, which were tested every six hours, were positive after 24 but not after 30 hours.

The whole blood samples kept in test tubes were positive after 160 but not after 190 days at 37°C, and after 190 but not after 224 days at room temperature. The blood kept in sealed pipettes was positive after 153 days (the longest period of test) at room temperature; it was positive after 153 but not after 180 days at 37°C. At

cold room temperature the material was positive after 221 days, the maximum period of test. Ostertag (1908) observed that blood of an infected animal remained virulent in the dried state for about 3 weeks and blood which was allowed to putrefy in a glass tube up to 100 days. Kraneveld and Djaenoedin (1935) reported that washings from blood kept on the walls of broken tubes were positive by the intramuscular inoculation of a rabbit up to 34 hours and that infected serum retained its virulence for 100 days (maximum period tested) when kept in tubes with paraffined cotton-wool plugs, but only 40 days in tubes plugged with dry cotton wool.

The virulence of the organism isolated from the blood kept in pipettes was tested at different stages in mice. The strain used originally had an MLD of one ml. of 10^{-8} dilution of a 24 hour broth culture. After isolation from the blood and maintenance at room temperature for 153 days the strain declined in virulence, the MLD being 10^{-4}. The strain isolated from the pipette kept at 37°C after a similar interval was fully virulent as was the strain isolated from the pipette kept in the cold room for 221 days.

Serial Passage in Carbon Dioxide

The first subculture on plain agar (C1) showed quicker growth than that on blood agar. The increased growth on plain agar was maintained up to the C11 generation; thereafter the growth on both the media was equal. Subcultures from plain agar (C1 to C11) when grown in the ordinary atmosphere showed early increased growth on plain agar as compared with blood agar under similar circumstances. Every time the proportion of carbon dioxide was increased, the first subculture showed a quicker and better growth. This phenomenon was observed in cultures C1 and C6 but was absent when the percentage of carbon dioxide was raised to 50 per cent. The reasons are not clear, but it is possible that growth of the organism is stimulated when first put in carbon dioxide as long as the carbon dioxide level is not too high. From generations C12 to C146 the rate of growth was normal both on plain agar and on blood agar.

Morphologically, there was no change up to generation C31 (Fig. 1): thenceforward the organism developed a monomorphic character, becoming slightly longer and the number of short filamentous forms also increased. The C78 subculture developed a large number of long filamentous forms (Fig. 2) which persisted up to C146, the maximum subculture studied. The filamentous forms gradually disappeared when the culture was incubated in carbon dioxide for more than 48 hours. On transferring to atmospheric conditions it was not possible to produce the filaments either on blood agar, plain agar or in plain broth. The variant exhibited no change in biochemical reaction. A change in the appearance of the colonies was noticed after 32 subcultures: they became rough, the summit of the usual cone was pitted, and the frill around its base as well as the radial striations from the top, which were the

characteristic features of the original strain, were absent. This variation in colony structure remained constant up to the last subculture studied.

In order to test the virulence of the variants, mouse pathogenicity tests were carried out with generations C10, C20, C33, C67, and C146. No change in virulence was noticed up to C20, but at C33 the MLD was 10^{-5} and remained at this level to C146, when the experiment was discontinued. It is interesting to note that the decrease in virulence started at the point when the colony character changed, together with the change in morphology of the organism.

Bacterial Antagonism to P. septica

In association with *B. anthracis,* Pasteurella were present in large numbers up to 144 hours; they subsequently steadily declined and after 244 hours, the maximum period tested, they had fallen considerably. The population of anthrax bacilli appeared to remain more or less stationary, although no quantitative test was carried out. In the presence of *B. subtilis,* Pasteurella were absent after 216 hours. In mixtures of Pasteurella with Staplylococci, Streptococci and Corynebacteria respectively, both Pasteurella and the test organism were present after 71 days, the longest period for which the materials were examined. *P. septica* isolated from all these mixtures was fully virulent for mice. Where *Bact. coli* was associated with Pasteurella organisms both were present after 23 days, but both had disappeared from the mixture after 43 days. In the presence of Pseudomonas organism, *P. septica* disappeared between the fifth and the seventh day.

It would thus appear that *Ps. pyocyanea* has a very strong antagonistic action on *P. septica.* Next comes *B. subtilis* followed by *B. anthracis.* The behaviour of *Bact. coli* appears to be peculiar in that both disappeared simultaneously after 43 days. No change was noticed in the biochemical and morphological characters of the Pasteurella strains isolated at various intervals from these combinations.

DISCUSSION

It is well known that growth of *P. septica* even on enriched media is not profuse. As a result, primary isolation of this organism from field material is often made difficult by more rapidly growing contaminants, the most troublesome of which is a spreading variety of Proteus. The medium described in this article checks the growth of contaminants. Some workers (Bain, 1956) advocate the incorporation of antifungal agents, but in the experience of the present author fungi have not complicated the isolation of *P. septica.*

Although the method of choice for the identification of *P. septica* is serotyping it cannot be adopted as a routine practice. Fermentation tests still have a place although unfortunately, the results reported by different workers have revealed significant divergences.

Russa (1939) observed that 11 strains fermented glucose, galactose, laevulose and mannite, and that sorbite was almost specific. According to Wilson and Miles (1948) glucose, mannite, sucrose and sorbite are fermented by all strains. Merchant (1953) states that only some strains produce acid in sorbite. Roberts (1947), however, designated as typical only those strains which fermented glucose, sucrose and mannite. It is obvious, therefore, that the results of fermentation tests are variable and so until definite information on the reasons for such wide variation is available any grouping based on fermentation is likely to be inaccurate. In the present study of the strains collected in India, galactose was more often fermented than mannite. If, therefore, an Asian strain is to be judged as 'typical' on the basis of sugar reaction, galactose, and not mannite, should be taken into account.

Viability of *P. septica* under various climatic conditions outside the body is bound to play a very important role in the spread and perpetuation of the disease. Blood and other materials from cases of pasteurellosis may remain infective for a very long period. The deciding factor seems more likely to be the moisture content of the material rather than the temperature, which may vary from 37°C to −4°C.

The experiment of serially passaging *P. septica* through an atmosphere of carbon dioxide was undertaken mainly to see if a fully antigenic attenuated strain could be evolved. Although the organism showed some sign of attenuation in the early stages its virulence was still too high for use as a live antigen.

The work on the antagonism of certain common contaminants was undertaken to see if the presence of these organisms in pathological specimens from haemorrhagic septicaemia cases would affect the viability of *P. septica* and thus throw some light on frequent reports of failure to isolate Pasteurella from suspected material. It seems that the presence of Pseudomonas and *B. subtilis* in the specimen may kill off Pasteurella within a short time. In addition, *B. anthracis* has an antagonistic action, an indication that a simultaneous infection with both Pasteurella and Anthrax may not be possible.

CONCLUSIONS

The results of some observations on certain biophysical characters of *P. septica* are reported.

A new selective culture medium has been evolved for the isolation of *P. septica*.

Forty-nine strains collected from field cases of Pasteurellosis in cattle, buffalo, sheep, goats and poultry were tested for sugar fermentation.

The viability of the organism in blood was tested on various substrates at room temperature, 37°C and at cold room temperature.

Antagonism of certain common contaminating bacteria, such as

B. subtilis, Streptococcus, Staphylococcus, Corynebacterium and *Bact. coli* against *P. septica* has been studied.

The behaviour of *P. septica* after serial growth in gradually increasing atmospheres of carbon dioxide is reported.

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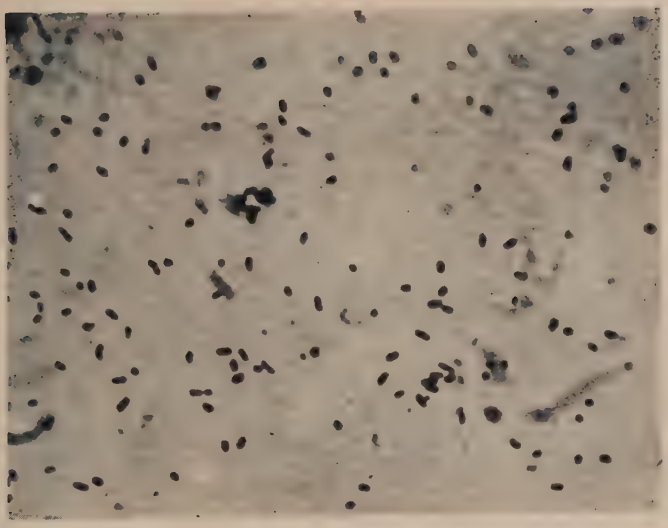
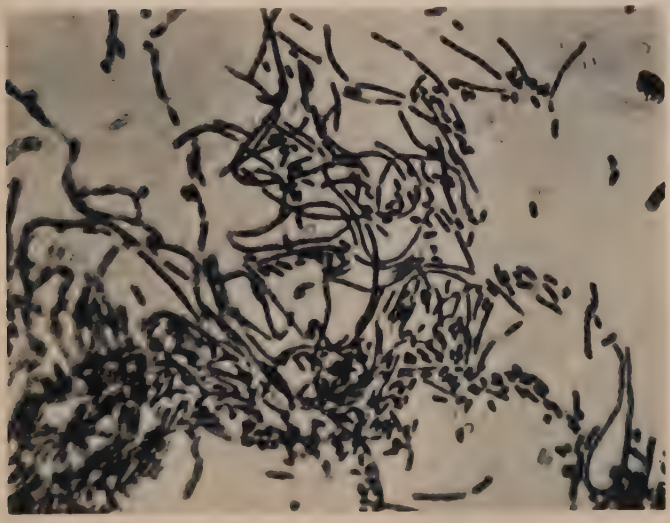
Fig. 1



Typical morphology of *P. septica* grown in air on selective medium.

Fig. 2



Long filamentous form in subculture C78 in 100 per cent carbon dioxide.

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THE ACTION OF BERENIL ON TRYPANOSOMES, INCLUDING STRAINS RESISTANT TO ANTRYCIDE AND TO STILBAMIDINE

By

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INTRODUCTION

Berenil is a new compound which has been introduced by Hoechstag for the treatment of trypanosomiasis and babesiasis in domestic animals (Milne, Robson and Lwebandiza, 1955; Bauer, 1955; Enigk and Reusse, 1955). Chemically it is pp′-diamidinodiazoaminobenzene diaceturate trihydrate $NH_2(NH).C_6H_4.N=N—NH.C_6H_4.(NH)\ NH_2+3H_2O$. Thus it is chemically related to stilbamidine ($NH_2(NH).C_6H_4.CH{:}CH.C_6H_4.(NH)\ NH_2$) and other diamidine compounds such as pentamidine and propamidine.

The present paper reports a laboratory investigation on its type of trypanocidal action, and on its action upon trypanosomes resistant to antrycide and to stilbamidine respectively.

MATERIALS AND STRAINS

The Berenil used was kindly supplied by Farbwerke Hoechstag. Fresh solutions were made for each experiment. The trypanosomes consisted of *T. equiperdum* (the standard strain which has been maintained at this Institute for over 20 years), an antrycide-resistant substrain of this (history given below), a stilbamidine-resistant strain of *T. rhodesiense* (see below) and *T. congolense*. All these were old laboratory strains, highly virulent for mice, killing them 3 to 6 days after intra-peritoneal inoculation.

The antrycide-resistant strain was made in 1952 from our stock strain of *T. equiperdum* by exposing it in vivo to repeated subeffective doses of antrycide. After six months of such treatment (i.e. December 1952) an intraperitoneal dose of 0·02 mg. per g. produced no effect upon infections in mice, while the parent strain was temporarily cleared from the blood by 0·0025 mg. per g., but not by 0·001 mg. This is the strain described by Hawking and Thurston (1955). The strain was then maintained by passage through mice without any further exposure to antrycide. When examined again in October 1956 (as described below) it was found to have lost part but not all of its resistance; it resisted 0·0025 mg. per g. but not 0·005 mg. while the normal strain was now cleared by 0·0006 mg. The strain was then passed through mice treated with antrycide. By June 1957 the trypanosomes resisted 0·005 mg. per g. i.p. and sometimes 0·01 mg. (0·0025 mg. per g. cleared 0/4 mice; 0·005 mg. cleared 2/9 mice; 0·01 mg. cleared 6/9 mice; and 0·02 mg. cleared 2/3 mice).

The stilbamidine-resistant strain was the one prepared by Fulton and Grant (1955) who reported that in mice it resisted the maximal tolerated dose of drug (4 × 1·0 mg. per mouse). This strain was also described by

Williamson and Rollo (1952). It was kindly provided by Dr. J. D. Fulton. When tested by us in 1956 it resisted stilbamidine in doses of 0·1 mg. per g.

RESULTS

Action of Berenil in vitro. Berenil was tested for its trypanocidal action in vitro, according to the technique of Yorke and Murgatroyd (1930). At 35°C. with 20 hours exposure, the minimum trypanocidal concentration was approximately 1 in 1·6 to 3·2 million for *T. rhodesiense* and for *T. equiperdum*, and about the same for *T. congolense*. When a rabbit was given 40 mg/kg. i.m. and the blood was withdrawn 6 hours later, the minimum trypanocidal titre of the plasma for *T. rhodesiense* (after 24 hours at 35°C.) as tested by the technique of Murgatroyd, Russell and Yorke (1934) was only 1:1, which corresponded to a concentration of Berenil of about 1 in 1·6 million; plasma taken at 24 hours usually had no trypanocidal action in vitro.

Action in vivo. The minimum effective dose (ED 80) of Berenil in our experiments was approximately 0·04 mg. per g. i.p. for mice infected with *T. equiperdum*, and 0·005 to 0·002 mg. per g. for our strain of *T. congolense*. (With stilbamidine the dose was 0·01 mg. for *T. equiperdum* and about 0·025 mg. for *T. congolense;* with pentamidine 0·05 mg. per g. was ineffective against *T. congolense*.) Thus Berenil is particularly active against *T. congolense*, while stilbamidine is particularly active against *T. equiperdum*. In animals infected with *T. equiperdum* or *T. congolense* the trypanosomes began to be killed in about 6 hours after the injection of large doses of Berenil; but after small doses the trypanosomes might multiply for 24 hours, and persist for 48 hours before they eventually disappeared. In this respect, Berenil resembles stilbamidine. The action of Berenil in vivo is thus less rapid than that of the trivalent phenyl arsenicals but more rapid than that of suramin, phenanthridine compounds, etc. When trypanosomes are exposed to Berenil in mice for 2 to 4 hours and are then injected into other mice, they are still infective in contrast to suramin and phenanthridines.

Action on trypanosomes resistant to stilbamidine or to antrycide. The drugs were given as a single dose intraperitoneally in mice. The mice were treated when the blood contained 1 to 200 trypanosomes per 20 high power microscope fields and they were examined for five successive days. With some drugs the blood was not cleared of trypanosomes until after 3 to 4 days. On any one occasion 3 mice were employed for each dose. The results of nine different experiments are presented in Table 1. Berenil was moderately active against our normal strain of *T. equiperdum* (MED, ·04 mg. per g.) but it was highly active against our antrycide-resistant strain (MED ·0012 mg. per g.); it had no action upon the stilbamidine-resistant strain of *T. rhodesiense*, which fact confirms that Berenil acts like a typical diamidine drug. The sensitivity of the antrycide-

TABLE 1

THE REACTION OF NORMAL, ANTRYCIDE-RESISTANT, AND STILBAMIDINE-RESISTANT TRYPANOSOMES TO BERENIL AND OTHER COMPOUNDS

Compound	*Dose mg./g i.p.*	*Percentage of mice cleared*		
		Normal (T. equiperdum)	*Antrycide-resistant* (T. equiperdum)	*Stilbamidine-resistant* (T. rhodesiense)
Berenil	·0006	0 (9)	44 (9)	
	·0012	0 (3)	100 (6)	
MTD 0·1 mg.	·0025	0 (3)	100 (6)	
	·005	0 (5)	80 (15)	0 (3)
	·01	0 (6)	100 (6)	
	·02	0 (3)	100 (6)	0 (3)
	·04	100 (6)	100 (6)	0 (9)
	·08	100 (3)		0 (3)
		MED ·04	MED ·0012	Resistant
Antrycide	·00015	0 (4)		
	·0003	22 (9)		
MTD ·015 mg.	·0006	100 (16)	0 (3)	50 (6)
	·0012	100 (12)	0 (7)	50 (6)
	·0025	100 (10)	15 (19)	100 (6)
	·005	100 (16)	43 (21)	
	·01		83 (18)	100 (3)
	·02		67 (3)	
		MED ·0006	MED ·01	MED ·0025
Stilbamidine	·0015	0 (3)	0 (3)	
	·003	0 (3)	0 (3)	
MTD 0·1 mg.	·006	0 (3)	67 (3)	
	·0125	100 (3)	100 (3)	
	·025	100 (3)	100 (3)	
	·05	100 (3)	100 (3)	
	·1	100 (6)	78 (9)	0 (3)
		MED ·0125	MED ·0125	Resistant
Dimidium	·025		0 (3)	
	·05		0 (3)	
MTD 0·1 mg.	·07	0 (8)	100 (9)	0 (9)
		Resistant	MED ·07	Resistant
Acriflavine	·015	33 (3)	83 (6)	0 (3)
	·02	100 (6)	83 (6)	0 (5)
MTD ·02 mg.		MED ·02	MED ·015 (or less)	Resistant
Tryparsamide	·5	55 (9)	67 (9)	0 (6)
	1·0	100 (9)	90 (9)	0 (9)
MTD 4·0 mg.	2·0			0 (3)
	4·0			0 (3)
		MED 1·0	MED 1·0	Resistant
Suramin	·00025		0 (3)	0 (3)
	·0005		0 (3)	0 (3)
MTD 0·5 mg.	·0015	11 (9)	65 (11)	100 (9)
	·003	33 (6)	100 (6)	100 (3)
	·005	100 (3)		
		MED ·005	MED ·003	MED ·0015

MTD = Maximum tolerated dose. MED = Minimum effective dose, mg./g.
The figures in brackets indicate the number of mice.

resistant strain to Berenil is interesting; but resistant strains vary greatly, and too much significance must not be attached to a single example.

Analysing the reactions according to strains, the antrycide-resistant strain was sensitive to Berenil (highly), stilbamidine, dimidium, suramin (? increased), acriflavine and tryparsamide. The stilbamidine-resistant strain was sensitive to suramin (markedly) and antrycide (moderately) and resistant to Berenil, stilbamidine, acriflavine and tryparsamide. Ormerod (1952) found his antrycide-resistant strain of *T. equiperdum* sensitive to suramin and p-hydroxyphenylarsenoxide, partially resistant to acriflavine, and resistant to stilbamidine and dimidium. Williamson and Rollo (1952) reported the stilbamidine-resistant strain as sensitive to antrycide and suramin but resistant to stilbamidine, acriflavine and arsenicals.

DISCUSSION

Compounds used for the treatment of trypanosomiasis may be divided into two main groups according to their method of effecting a cure. In one group, which includes the trivalent arsenical compounds, acriflavine, pentamidine, etc., (a) the compound rapidly kills trypanosomes in vivo and in vitro; (b) it fails to prevent trypanosomes infecting sub-inoculated animals, apart from killing them before they are sub-inoculated; (c) it combines with the trypanosomes in large amounts.

In the other group, which includes suramin, ethidium etc., and antrycide, (a) the compound does not readily kill trypanosomes in vitro, while in vivo there is a lag period of 24 to 48 hours after the treatment before the trypanosomes are killed; (b) trypanosomes exposed to the compound are not infective for sub-inoculated animals, even though the trypanosomes appear actively motile before injection; (c) only small amounts of the compound combine with the trypanosomes.

The purpose of the present investigation was to discover the class of trypanocidal compounds to which Berenil belongs. The results show that it belongs to the first class of compounds (the trivalent arsenical, acriflavine, diamidine group), since it is actively trypanocidal in vitro and it does not make trypanosomes non-infective although still motile. Further its activity upon drug-resistant strains, i.e. its failure to act upon a stilbamidine-resistant strain, shows that it behaves like the other diamidine compounds which it resembles in chemical structure. The triazene chain (—N=N—N—) linking the two halves of the molecule does not alter the general characteristics of the diamidine group, although it renders Berenil particularly active against *T. congolense;* while stilbamidine and pentamidine are particularly active against *T. equiperdum* and *T. rhodesiense.* Presumably this difference of activity depends upon the difference in effective distance or of orientation between the active groups at

each end of the molecule, which reflects the distance apart of some corresponding receptor groups in *T. congolense* and in *T. equiperdum* respectively.

CONCLUSIONS

Berenil (pp'-diamidinodiazoaminobenzene diaceturate), a compound recently introduced for the treatment of trypanosomiasis and babesiasis in animals, is actively trypanocidal in vitro, killing laboratory strains of *T. rhodesiense*, *T. equiperdum* and *T. congolense* in a concentration of about 1:2 million at 35°C in 20 hours. When injected into rabbits intramuscularly the plasma is trypanocidal after 6 hours but not after 24 hours. In mice, it is more active against *T. congolense* than against *T. equiperdum*. The trypanocidal action takes 6 to 48 hours to manifest itself according to the dose, and during this period the infectivity of the trypanosomes for fresh mice is preserved.

An antrycide-resistant strain of *T. equiperdum* was highly sensitive to Berenil, but a stilbamine-resistant strain of *T. rhodesiense* was completely resistant. The reactions of these strains to other drugs were also investigated.

The trypanocidal action of Berenil resembles that of the other diamidine compounds, which it also resembles in chemical structure.

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THE EFFECT OF NICARBAZIN ON GROWTH RATE, SEXUAL MATURITY, EGG PRODUCTION, FERTILITY AND HATCHABILITY

By

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INTRODUCTION

Nicarbazin, a molecular complex of 4:4′-dinitrocarbanilide and 2-hydroxy-4:6-dimethylpyrimidine is used as a coccidiostat for growing chicks.

The anti-coccidial activity of Nicarbazin has been reported by several workers (Barber, 1955; Cuckler and Malanga, 1955; Fogg and Siegmund, 1955; Cuckler, Malanga, and Ott, 1956). Ball (1956), in these laboratories has confirmed its effectiveness.

Ott, Kuna, Porter, Cuckler and Fogg (1955) found that Nicarbazin, when administered in the feed at levels between 0·01 per cent and 0·02 per cent w/w permitted optimal growth and did not interfere with sexual maturity, egg production or fertility in adult chickens. Sherwood, Milby, and Higgins (1956) failed to show any effect on fertility, but found that hatchability was reduced to approximately 50 per cent by Nicarbazin at concentrations between 0·005 per cent and 0·0125 per cent.

Loss of pigment in egg shells following administration of Nicarbazin to laying hens has been reported by McClary (1955), Ott, Dickinson, and Peterson (1955), and Sherwood *et al.* (1956).

As Nicarbazin is now being used in the United Kingdom, it was felt necessary to obtain further information on the effect of feeding it, at the recommended coccidiostatic level (0·0125 per cent), on growth-rate, egg production, hatchability, and fertility.

MATERIALS AND METHODS

Chickens. Day old, sexed chicks obtained from a flock of White Leghorns kept by the Animal Health Trust at Houghton Grange, were reared in wire floored, heated cages. At nine weeks of age they were moved outside into verandahs with wooden lath floors. At seventeen weeks the pullets were placed in laying batteries, one bird to each cage, and the cockerels into individual cages. Care was taken to distribute the birds so that light, heat, and air conditions were spread as evenly as possible throughout the groups.

Diet. Proprietary meal was fed throughout. Medicated meal was prepared in a mechanical mixer, using "Nicoxin", a 25 per cent w/w premix of Nicarbazin, to give a concentration of 0·0125 per cent w/w Nicarbazin. Food and water were available *ad lib.* Samples of medicated food were taken at intervals and the Nicarbazin content determined.

Experimental groups. The day old chicks were divided into groups, wing banded and weighed. The pullets were divided into 6 groups, A to F. Groups A and B, each of 24 birds, received unmedicated food and acted as controls. Groups C and D, again each of 24 birds, were fed

medicated meal for the whole period of the trial, i.e. until the birds had been in lay for five months. Group E, 12 birds, was fed medicated meal from 1 day to 13 weeks of age, followed by unmedicated meal, and Group F, 16 birds, received unmedicated food until 19 weeks old and then medicated meal. The cockerels were contained in two groups (20 birds in each), one group as a control for the other group which received medicated meal throughout.

Experimental procedure. The birds were weighed individually at intervals. All eggs produced including soft shelled and broken eggs were recorded. On certain days throughout the experiment all the eggs produced were weighed. Yolks were examined physically for signs of uneven texture. Estimations of Nicarbazin in the whites and yolks were made. A random selection of eggs, bearing the hen number, was incubated, cracked eggs and double-yolked eggs being discarded. Each batch of eggs was candled after seven days incubation, the 'clears' were removed and broken open to examine for fertility. All eggs remaining unhatched after 22 days were examined and the age of the embryo at death recorded.

Fertilisation of the pullets was by artificial insemination with pooled semen collected from all the cockerels within a group. Laying pullets were inseminated twice weekly with 0·05 ml. of fresh undiluted semen. Pullets in Groups A and C were inseminated with semen from cockerels in the control group, and those in Groups B and D with semen from the cockerels which were receiving the medicated diet. Groups E and F were inseminated with semen from the control cockerels.

TABLE 1

EFFECT OF NICARBAZIN ON INCREASE IN WEIGHT

Group	*Number of birds*	*Nicarbazin % w/w in feed*	*Mean weight (g) at*			
			1 day	*20 days*	*11 weeks*	*11 months*
A	24	Nil	35	144	836	1869
B	24	Nil	35	144	850	1839
C	24	0·0125	35	141	863	1923
D	24	0·0125	34	135	849	1999
Cockerels	20	Nil	41	144	965	2497
Cockerels	20	0·0125	41	149	1025	2537

RESULTS

Estimation of the Nicarbazin concentration in samples of medicated diet gave results between 0·0120 per cent and 0·0130 per cent (Mean 0·0128 per cent). The effect of feeding Nicarbazin on weight gain is shown in Table 1. The mean weight of pullets at 11 months, in Groups A and B, was 1854 g. (S.E. ±36·3), and in the medicated groups, C and D, was 1961 g. (S.E. ±45·2). The

difference between the means is significant at P equals or is not greater than 0·05. The difference between the means of the two groups of cockerels, 2497 g. (S.E. ±63·04) and 2537 g. (S.E. ±89·7), was not significant at the same probability level.

Maturity

The maturity of the pullets, as indicated by the age at which laying commenced, was not affected by medication of the diet. The sudden introduction of Nicarbazin into the diet at 19 weeks of age, when the birds were near to point of lay, (Group F, Table 2) did not appear to cause any delay in production. Table 2 shows the age at which the first egg was laid.

TABLE 2

COMMENCEMENT OF LAY

Group	*Number of birds*	*Nicarbazin % w/w in feed*	*Age in weeks*						
			19	*20*	*21*	*22*	*23*	*24*	*25*
			Number of birds in lay						
A	24	Nil	—	1	5	13	20	21	22
B	24	Nil	—	1	8	11	15	16	18
C	24	0·0125	—	1	7	12	14	18	22
D	24	0·0125	2	3	8	12	19	22	23
E	12	0·0125 (0 to 13 wks.)	—	—	1	3	7	8	11
F	16	0·0125 (from 19 wks.)	1	—	3	8	12	13	15

Egg Production

The effect of feeding Nicarbazin on egg production is shown in Table 3. The mean number of eggs laid by each bird in groups A and B (unmedicated diet) was 60·52 (S.E. ±3·29) and in groups C and D, (medicated diet) was 50·58 (S.E. ±2·85). The difference between the means is significant ($P<0.05$). The mean number of eggs laid by each bird in Group E was 55·17 (S.E. ±6·79) and by the birds in Group F was 57·88 (S.E. ±5·44); the mean difference between each of these groups and the controls, groups A and B, is not significant. Mean egg production was calculated from the records of all the birds in each group that survived to the end of the laying test period, whether the birds were in lay or not.

Egg Weight

Egg weight was not affected to a significant degree by the administration of Nicarbazin. In Table 4 the average weights of

all eggs laid on certain days by birds in each group are recorded. Fresh eggs were broken open and examined for mottling of the yolk. Mottling takes the form of clear patches in the substance or on the surface of the yolk and is believed to be due to the absorption of albumin into the yolk material (Scholes, 1956). Examination was made on the same day that the eggs were laid. Mottled yolks were found in 66 per cent of the eggs from the birds receiving medicated diet. Ten per cent of the eggs from the control pullets had yolks of uneven texture, insufficiently differentiated to be designated as mottled. Estimation of the components of Nicarbazin in the yolks and white of eggs from pullets receiving medicated diet indicated the presence in the yolks of the dinitrocarbanilide component at concentrations between 14·5 and 11·75 μg/g., with a mean value of 13·41 μg/g.

TABLE 3

EFFECT OF NICARBAZIN ON EGG PRODUCTION

Group	*Number of birds*	*Nicarbazin % w/w in feed*	*Mean egg production/bird*				*Mean total eggs/bird*
			June July	*August*	*September*	*October*	
A	23[1]	Nil	17	21	16	9	63
B	21[2]	Nil	15	18	16	9	58
C	24	0·0125	13	18	12	5	48
D	24	0·0125	14	18	14	7	53
E	12	0·0125 (0 to 13 wks.)	9	17	17	13	55
F	16	0·0125 (from 19 wks.)	16	17	15	9	58

1. One bird died of egg peritonitis and is not included.
2. One bird died of egg peritonitis and 2 birds from leucosis and are not included

Fertility and Hatchability

The effect of Nicarbazin on the fertility of pullets and cockerels is shown in Table 5. The fertility of the pullets was reduced by at least 50 per cent. There was no significant difference in the fertility of the two groups of cockerels, although the lowest percentage fertility in the pullets occurred when both pullets and cockerels were receiving the medicated diet (Group D).

Hatchability of fertile eggs (Table 5) was reduced by medication of the pullets. Medication of the cockerels, alone, did not effect hatchability.

Embryo Mortality

The incidence of embryo mortality (Table 6) was highest in the first seven days of incubation in groups D (46 per cent), C (40 per cent) and F (42 per cent). In the unmedicated groups (A, and B) mortalities were dispersed more evenly between the three weeks of incubation. Chicks hatched and kept for seven days showed no difference in mortality between groups.

TABLE 4

EFFECT OF NICARBAZIN ON EGG WEIGHT

Group	*Nicarbazin % w/w in feed*	*Number of eggs weighed*	*Average weight (g).*
A	Nil	187	51·65
B	Nil	165	49·22
C	0·0125	175	51·83
D	0·0125	179	51·82
E	0·0125 (0 to 13 wks.)	77	46·79
F	0·0125 (from 19 wks.)	117	50·59

TABLE 5

EFFECT OF NICARBAZIN ON HATCHABILITY AND FERTILITY

Cockerels	*Pullets*		*Number of eggs (total)*			*Percentage fertility*	*Percentage fertile eggs hatched*
	Group	*Nicarbazin (%)*	*Incubated*	*Fertile*	*Hatched*		
Medicated	B	Nil	288	288	186	100	65
	D	0·0125	283	66	23	23	35
Unmedicated	C	0·0125	284	119	67	42	56
	A	Nil	283	238	192	84	81
	E	0·0125 (0 to 13 wks.)	157	128	94	82	74
	F	0·0125 (from 19 wks.)	253	116	57	46	49

TABLE 6

EMBRYO MORTALITY

Cockerels	*Pullets*		*Embryo deaths*			
	Group	*Nicarbazin % w/w in feed*	*Incubation week*			*Total*
			1	*2*	*3*	
Medicated ..	B	Nil	13	15	14	42
	D	0·0125	20	5	18	43
Unmedicated ..	C	0·0125	21	13	18	52
	A	Nil	15	13	18	46
	E	0·0125 (0 to 13 wks.)	11	6	17	34
	F	0·0125 (from 19 wks.)	25	10	24	59

DISCUSSION

Nicarbazin fed in the meal at the concentration recommended for the prevention of coccidiosis, i.e. 0·0125 per cent w/w, did not affect the weight of White Leghorn chickens up to 11 weeks of age. At 11 months the pullets receiving medicated diet were significantly heavier than the control pullets. Barber (1955) found no depression of weight when Nicarbazin was fed at 0·02 per cent to R.I.R. chicks from 3 to 9 weeks of age, and Fogg, Cuckler, Ott and Siegmund (1956) fed concentrations of Nicarbazin up to 0·04 per cent for 3 or 11 weeks in environments containing and free from coccidia and found that chickens grew normally. Fogg and Siegmund (1955) concluded as a result of field trials involving 90,840 broilers, that Nicarbazin permits increased growth. Although Harwood, Stunz and Wolfgang (1956) found that Nicarbazin fed continuously at 0·0125 per cent to White Leghorn chicks until they were 4 weeks of age retarded growth rate to a highly significant degree, they concluded that their results "are not contrary to the findings of other workers as their experimental chicks were disease-free and were reared on wire mesh floors."

Sexual maturity, as indicated by the onset of egg production was not affected by Nicarbazin. This is in agreement with the findings of Ott *et al.* (1955) who found sexual maturity to be normal in birds receiving concentrations between 0·01 per cent and 0·02 per cent. Depression of egg production occurred in birds that received the medicated diet throughout life. There was, however, no effect on subsequent egg production when Nicarbazin was fed for the first 13 weeks of life only. Sherwood, Milby and Witz, (1956) report

that White Rock Breeder hens, one and two years old, produced fewer eggs when fed Nicarbazin in the diet.

Fertility and hatchability were reduced by Nicarbazin in the diet. The greater reduction occurred when both pullets and cockerels were on medicated diets. This effect may be peculiar to White Leghorns as Sherwood, Milby and Higgins (1956) found no consistently adverse effect on fertility but a fall in hatchability by 50 per cent in one and two year old White Rock birds.

Mottling of egg yolks following feeding of Nicarbazin has been reported by Polin and Porter (1956) who found a direct relationship between the degree of mottling and the concentration of Nicarbazin in the diet.

CONCLUSIONS

Nicarbazin, mixed in proprietary mash to give a concentration of 0·0125 per cent w/w and fed to White Leghorn chickens, was without adverse effect on growth rate and sexual maturity.

When fed for the first 13 weeks of life, Nicarbazin produced no adverse effect on subsequent egg production, fertility or hatchability.

When Nicarbazin was fed to laying birds, production, fertility and hatchability were reduced and mottling of egg yolks occurred.

Administration of Nicarbazin should be confined to birds during the rearing period, beyond which adverse effects are produced.

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A NEUTRALIZATION TEST FOR THE DETECTION OF RINDERPEST ANTIBODIES

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INTRODUCTION

During the past four years we have studied, independently at Kabete and Muguga, rinderpest immunity and found that the most useful technique was the detection of neutralizing antibodies. We developed standard procedures which differed only in minor detail and which were based on the system by which Nakamura (1940) followed the development of antibodies in rabbits given lapinised rinderpest virus. The techniques and applications are herein recorded.

MATERIALS AND METHODS

Virus. The test virus was the Kabete substrain of the Nakamura III strain of lapinised rinderpest virus which induces in inoculated rabbits a characteristic thermal reaction and pathognomonic lesions in the lymphoid tissues (Fukusho and Nakamura, 1940). The virus was stored at minus 20°C as a freeze-dried suspension of infected rabbit tissues. The titre in rabbits was $10^{5.0 \pm 0.3}$ fifty per cent infective doses (RID_{50s}) per gramme of tissue and was not lowered significantly after a year's storage. Thereafter fresh stocks of virus were prepared.

Sera. Cattle, goat, rabbit, hamster, mouse and dog sera were examined.

In cattle five regimens of antibody response were investigated. First, sera were taken before and 21 days after the inoculation of four strains of virulent rinderpest virus; the Kabete 'O' (Montgomery, 1910), the Kabete hamster (Scott, unpublished), the Muguga mouse (Scott, unpublished) and the Muguga tissue culture strains (Plowright and Ferris, 1957). Secondly, sera were examined at varying intervals after inoculation with four strains of attenuated rinderpest virus; the Kabete caprinised (Daubney, 1937), the Nakamura III lapinised (Nakamura, Wagatuma and Fukusho, 1938), the Japanese avianised (Ishii, Furuya and Takemoto, 1952), and the Nakamura avianised-lapinised strains (Nakamura and Miyamoto, 1953). Thirdly, sera were taken from cattle given seven types of partially-inactivated rinderpest vaccine; formalinised bovine spleen, formalinised bovine spleen adsorbed on to aluminium hydroxide, formalinised bovine spleen combined with a mineral oil adjuvant, formalinised bovine spleen combined with an adjuvant of mineral oil and killed *Mycobacterium butyricum*, formalinised tissue culture virus, formalinised tissue culture virus adsorbed on to aluminium hydroxide and formalinised tissue culture virus combined with a mineral oil adjuvant. Antibodies associated with naturally-acquired passive immunity were sought in the sera of calves, born of immune dams, after suckling, and in the dam's serum, colostrum and milk. Finally, we examined the sera of cattle inoculated with rinderpest immune serum.

The immune response of inoculated and vaccinated cattle was confirmed by challenging survivors with either the Kabete 'O' or the caprinised strains of rinderpest virus, together with known susceptible control cattle.

The goat sera were collected at varying intervals after the inoculation of two virulent strains of virus, the Kabete 'O' and the Muguga mouse strains. Similarly sera were taken from goats after inoculation with the attenuated caprinised and lapinised strains. The rabbit sera came from rabbits inoculated intravenously with the lapinised and avianised-lapinised viruses. The hamster sera were collected from hamsters previously infected with the hamster strain of rinderpest. Mice previously infected with the mouse strain were bled for serum. The dog sera were withdrawn from dogs inoculated with the Kabete 'O' strain of the virus. Confirmation of viral multiplication in the goats, hamsters, mice and dogs was obtained by demonstrating the infectivity for cattle of blood taken between the 4th and 8th day after inoculation. Multiplication of the lapinised virus in the rabbit was shown by the development of pathognomonic lesions in the lymphoid tissues. The immunological response of rabbits given avianised-lapinised virus was further tested by challenging the rabbits with the lapinised strain, together with known-susceptible control rabbits.

Rabbits. Various breeds of European rabbits (*Oryctolagus cuniculus*) reared in Kenya were used when 4 months of age.

Standard neutralisation test. The sera were inactivated at 56°C for 30 minutes. Thereafter serial fivefold (Muguga) or tenfold (Kabete) dilutions were prepared in "Lemco" broth, pH 7·3. Equal volumes of a dilution of stock lapinised rinderpest virus, calculated on the basis of previous titrations to contain 20 to 200 RID_{50s} were added to each serum dilution to give final serum dilutions of either 1:2, 1:10, 1:50, 1:250, 1:1,150 and 1:6,250 (Muguga) or 1:10, 1:100, 1:1,000 and 1:10,000 (Kabete). The mixtures were shaken and held either at 37°C for one hour (Muguga) or at 4 to 10°C for 20 hours (Kabete). After incubation, 1·0 ml. amounts of each mixture were inoculated intravenously into 5 rabbits which were killed five days later and examined for the lesions of lapinised rinderpest infection. Both the Kabete and Muguga test included a control titration of the virus used. The Muguga test had a further control in which dilutions of a rinderpest-immune serum of known antibody titre were mixed with 20 to 200 RID_{50s} virus. Control serum-virus mixtures were handled similarly to the unknown. Consequently, at Kabete, each unknown serum required 21 to 35 rabbits. At Muguga, 33 to 55 rabbits were used. Normally several unknown sera were tested simultaneously to economise in the number of control rabbits.

Serum titres (SN_{50s}) were calculated at Kabete by the Reed and Muench formula (1938) and at Muguga by Thompson's method (1947). The Muguga titres were corrected by comparison with that of the standard rinderpest-immune serum. For convenience, the titres were expressed as the reciprocals of the logarithms of the 50 per cent end-point serum dilutions.

Test limits. Checkerboard titrations of known-immune serum in serial dilutions of the virus were made and replicated. The relationship between the amount of antibody and the quantity of virus neutralized was calculated by the method of least squares. The results were tested for significance by the Analysis of Variance. Variation between replicates

was calculated from the titres of the Muguga standard rinderpest-immune serum, which were estimated at frequent intervals over a period of twelve months.

Type of incubation. Serum-virus mixtures were incubated either for 20 hours at 4 to 10°C or for one hour at 37°C. The titres were compared and examined for significant differences by Student's 't' test.

RESULTS

Detection of Antibodies

Antibodies were not found in the sera of rinderpest-susceptible animals. Antibodies which neutralized lapinised rinderpest virus were detected in the sera of all the species in which multiplication of the virus occurred (Tables 1 and 2). The geometric mean titre, together with the 95 per cent fiducial limits, 21 days after inoculation was $3{\cdot}1 \pm 0{\cdot}3$. Differences between the titres of the sera of different species were not significant ($F = 3{\cdot}17$, $P < 0{\cdot}10 > 0{\cdot}05$). Likewise the differences attributable to the strains of virus were not significant ($F = 2{\cdot}32$, $P < 0{\cdot}20 > 0{\cdot}10$). In cattle, the pathogenicity of the virus used had no effect on the SN_{50} at 21 days after inoculation ($F = 1{\cdot}24$, $P > 0{\cdot}20$). Antibodies were detected in cattle sera 5 days after inoculation with KAG but not at 4 days and persisted for at least 13 years.

TABLE 1

NEUTRALISING ANTIBODY TITRES

Sera of cattle given viable rinderpest virus

Viral strain	*Serum neutralising titre*
Virulent strains	
Kabete 'O'	3·5
Kabete hamster	3·8
Muguga mouse	3·2
Muguga tissue culture	3·5
Attenuated strains	
Caprinised	3·0
Lapinised	3·7
Avianised	3·5
Avianised-lapinised	2·3

In the tables the titre is expressed as the reciprocal of the logarithm of the 50 per cent end-point dilution

Partially-inactivated vaccines also stimulated the production of antibodies (Table 3). Differences between the titres associated with different types of partially-inactivated vaccines were not significant ($F=0{\cdot}22$, $P>0{\cdot}20$). The number of doses of vaccine administered appeared to influence the subsequent titre, in that the greater the number of doses the higher the serum titre. The regression was not however significant ($F=2{\cdot}37$, $P<0{\cdot}20>0{\cdot}10$). The mean titre together with the 95 per cent fiducial limits was $2{\cdot}0 \pm 0{\cdot}6$, which was significantly lower than that stimulated by infectious virus ($t=4{\cdot}231$, $P<0{\cdot}001$).

TABLE 2

NEUTRALISING ANTIBODY TITRES

Sera of experimental animals inoculated with rinderpest virus

Animal	*Viral strain*	*Serum neutralising titre*
Goat	Kabete 'O'	3·0
	Muguga mouse	2·8
	Caprinised	3·3
	Lapinised	3·5
Rabbit	Lapinised	2·3
	Avianised-lapinised	2·0
Hamster	Kabete hamster	3·5
Mouse	Muguga mouse	2·5
Dog	Kabete 'O'	3·5

Naturally-acquired passive immunity was associated with antibodies in the sera of calves which had suckled immune dams. The mean titre, together with the 95 per cent fiducial limits, of six calves 30 hours after the ingestion of the colostrum was $2{\cdot}8 \pm 0{\cdot}3$. The mean titre of the dams' sera was $2{\cdot}4 \pm 0{\cdot}3$ and the mean titre of the colostrum was $3{\cdot}3 \pm 0{\cdot}2$. The antibody titre of the milk two days after parturition was $1{\cdot}3 \pm 0{\cdot}7$.

Antibodies associated with artificially-acquired passive immunity were related to the amount and titre of immune serum administered. For example, a steer weighing 650 lbs. had a titre of 0·7 when 100 ml. of immune serum with a titre of 3·2 was given, whereas an animal of similar weight given 1,000 ml. of the same serum had a titre of 1·3.

Variation between Replicate Antibody Titrations

The mean and 95 per cent fiducial limits of twenty estimations of the titre of a standard rinderpest immune serum was $2{\cdot}1 \pm 0{\cdot}1$.

The coefficient of variation was 12·4 per cent. The titre estimations had been made over a period of twelve months and the regression of the titres on time was not significant ($F=0{\cdot}14$, $P>0{\cdot}20$).

TABLE 3

NEUTRALISING ANTIBODY TITRES

Sera of cattle inoculated with partially-inactivated rinderpest vaccines

Type of vaccine	*No. of doses*	*Interval between doses (weeks)*	*Time of sampling after last dose (weeks)*	*Serum neutralising titre*
Formalinised bovine spleen	3	3	3	2·8
Formalinised tissue culture	2	3	3	1·3
Formalinised bovine spleen adsorbed on to Aluminium Hydroxide ..	1	—	4	1·0
Formalinised tissue culture adsorbed on to Aluminium Hydroxide ..	2	3	3	2·3
Formalinised bovine spleen combined with mineral oil	3	3	3	2·3
Formalinised tissue culture combined with mineral oil	1	—	4	1·5
Formalinised bovine spleen combined with mineral oil and killed *M. butyricum*	1	—	4	2·5

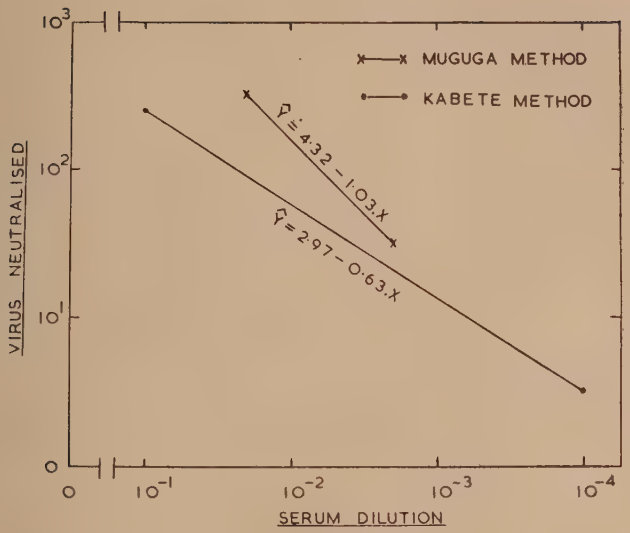
Relationships between Antibody and Virus

The regressions of lapinised rinderpest virus neutralised on the dilution of serum were calculated from checkerboard titrations carried out by the Kabete and Muguga techniques (Fig. 1). The regressions were linear, negative and significant ($\hat{Y}=2{\cdot}97-0{\cdot}63{\cdot}X$, $F=11{\cdot}82^{**}$, and $\hat{Y}=4{\cdot}32-1{\cdot}03{\cdot}X$, $F=4{\cdot}76^{*}$ respectively). The difference in the slopes of the lines was not significant ($t=0{\cdot}792$, $P<0{\cdot}5>0{\cdot}4$). Successful neutralisation occurred within the limits of $10^{0.0}$ to $10^{4.0}$ ID_{50s} of virus and $10^{-0.3}$ to $10^{-4.5}$ dilutions of serum.

Effect of the Type of Incubation

The similarity in the regression lines derived from checkerboard titrations by the Kabete and Muguga techniques suggested that the temperature and time of incubation had little effect on the subsequent antibody titre. This was confirmed by direct comparison (Table 4). The difference in the titres was not significant ($t=0{\cdot}476$, $P<0{\cdot}7>0{\cdot}6$). In other words, serum-virus mixtures incubated for one hour at 37°C gave similar results to mixtures incubated for 20 hours at 4 to 10°C.

Fig. 1



The relationship between the serum titre and the amount of lapinized rinderpest virus present in the serum-virus mixtures.

TABLE 4

NEUTRALISING ANTIBODY TITRES

Rinderpest-immune sera incubated with virus for one hour at 37°C and for 20 hours at 4—10°C

One hour at 37°C	*20 hours at 4—10°C*
3·4	3·3
2·7	2·5
2·9	2·5
2·8	3·0
3·3	3·0
3·0±0·4	2·9±0·4

DISCUSSION

Qualitative and quantitative detection of antibodies associated with all possible types of acquired immunity to rinderpest was possible with Nakamura's neutralisation system based on the

quantal response of rabbits to lapinised rinderpest virus. The main objection to the test is the high cost of the number of rabbits required. Nevertheless the rabbit test is more economical than either the usual challenge procedure in which immunity is checked by exposing cattle to virulent virus (Koch, 1897) or the neutralisation test described by Haddow (1935) in which cattle are used as the indicator hosts. The Nakamura III strain of lapinised rinderpest virus induced pathognomonic lesions if infective and as such gave clear-cut results which were not possible with the Baker strain of lapinised rinderpest virus used on Gross Isle (Baker, 1946; Jenkins and Walker, 1946).

CONCLUSIONS

A neutralisation test for the detection of rinderpest antibodies is described and is based on the response to lapinised rinderpest virus. Antibodies were detected in the sera of cattle with a naturally-acquired passive immunity, with an artificially-acquired passive immunity, with naturally-acquired active immunity and with an artificially-acquired active immunity. Virulent and attenuated strains of virus stimulated similar levels of antibody and these were significantly greater than those following vaccination with partially-inactivated virus. Antibody titres in other animals in which the virus multiplied were similar to the titres in cattle. The relationship between the amount of virus neutralised and the dilution of serum required was linear.

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STUDIES ON *PASTEURELLA SEPTICA*

II. Some Cultural and Biochemical Properties of Strains from Different Host Species

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INTRODUCTION

The frequent isolation of *Pasteurella septica* from the upper respiratory tract of healthy dogs has already been reported (Smith, 1955); during this study it was noticed that many of the strains from dogs differed from standard descriptions of the organism. To investigate these differences, strains from dogs, cats, pigs and cattle were examined by various methods and their properties compared. The results of certain cultural and biochemical tests are presented in this paper, and serological results will be reported later.

MATERIALS AND METHODS

Strains from healthy dogs were those obtained during the survey reported previously. Others were collected from healthy household cats by swabbing the mouth or throat. Further isolations were made from material received for routine bacteriological examination, and strains from cattle and pigs were obtained from slaughterhouse specimens. Blood agar was used for isolation, and cultures were regarded as *Past. septica* if they satisfied the criteria given in the previous paper; they were freeze-dried until required. A total of 103 strains was collected from sources detailed below.

Cattle. 5 strains (haemorrhagic septicaemia, 2; abortion, 1; arthritis, 1; calf pneumonia, 1).

Pigs. 28 strains (pneumonic lung, 26; diaphragmatic abscess, 1; healthy tonsil, 1).

Dogs. 39 strains (healthy nose or tonsils, 27; laryngitis or tonsillitis, 6; pneumonia. 3; mastitis, 1; blood culture in virus hepatitis, 1; blood culture in undiagnosed disease, 1).

Cats. 31 strains (healthy mouth or throat, 20; sepsis, 7; pleurisy, 2; blood culture during feline enteritis, 1; gingivitis, 1).

Capsule, colony iridescence and acid-agglutinability were studied because of the association of these characters with pathogenicity for mice and other animals (Priestley, 1936a, 1936b; de Kruif, 1922a, 1922b, 1922c). Hyaluronic acid production was examined in view of an important observation by Carter (1955) that this substance is produced by many mucoid strains of *Past. septica* and may interfere with certain serological procedures. Since many dog strains had been observed to have the unusual property of fermenting maltose, a wide variety of carbohydrate and other substances were used for fermentation tests.

Capsule demonstration. Jasmin's staining method (Jasmin, 1945) was found more satisfactory than the indian ink method described by Priestley (1936b). Smears from culture suspensions in 10 per cent serum

were stained with carbol fuchsin diluted 1:4 with water. Unstained zones around the bacteria were taken to indicate a capsule; the background of coagulated serum stained pink.

Iridescence. Strains were grown on 10 per cent horse serum agar plates for 24 hours. Iridescence was best seen when the plates were examined in strong sunlight falling obliquely on the plate from behind, the plate being held at such an angle that a shadowy part of the room formed a dark background.

Hyaluronic acid production. The total growth from a thickly sown 24 hour blood agar plate was suspended in 0·5 ml. saline. After centrifugation 0·3 ml. of the clear supernatant was mixed with 0·3 ml. of 10 per cent acidified horse serum which gives a white turbidity with hyaluronic acid (Seastone, 1943). After $\frac{1}{2}$ hour at room temperature the turbidity was measured by comparison with Brown's standard tubes and with four extra tubes corresponding to $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ and $\frac{1}{16}$ of the opacity of Brown's tube 1 (i.e. Brown 0·5, 0·25, 0·125 and 0·0625). The results were expressed as the turbidity attained, measured by this method.

Acid-agglutination. From a 24 hour peptone water cultures of each strain volumes of 0·3 ml. were mixed with equal volumes of McIlvaine's phosphate-citric acid buffers at pH 2·5, 3·5, 4·0, 4·5 and 5·5 in small tubes and incubated 18 hours at 55°C before examining for agglutination.

Mouse pathogenicity. Two 15 to 20 g. mice were each given 0·5 ml. intraperitoneally of a 24 hour broth culture and two received 0·5 ml. of a 1/1000 dilution of the same culture (0.0005 ml). Most deaths occurred within 48 hours but the mice were observed for 14 days. Results were recorded as — = no deaths; + = one or both mice receiving 0·5 ml. died but those receiving 0·0005 ml. survived; ± - = one or both mice receiving 0·0005 ml died.

Carbohydrate fermentation. Solutions of the various substances (1 per cent) in peptone water with Andrade's indicator were used. The tubes were incubated for 14 days and examined at intervals.

RESULTS

Morphology

All strains were Gram negative rods or coccobacilli; there were slight differences between strains from the different host species. Strains from cattle were non-pleomorphic and about 0·5 × 1·2 μ in size. Strains from pigs were non-pleomorphic and almost coccal, about 0·8 × 1·0 μ; nearly all pig strains possessed a capsule or envelope which, although invisible in Gram stained smears, tended to produce a very even distribution of organisms, each individual being separated from its neighbours by a capsule layer of even thickness. Strains from dogs were varied: a few resembled the bovine strains, some were exceedingly small coccobacilli about 0·4 × 0·7 μ in groups or short chains, while others were highly pleomorphic with large curved or S-shaped forms up to 1·2 × 5·0 μ and globular forms up to 1·4 μ diameter; it was usual for a proportion of these swollen involution forms to stain only very

palely with safranin. Strains from cats resembled those from cattle but tended more towards pleomorphism.

Table 1 summarizes the findings as regards capsule, formation of mucoid colonies, iridescence, hyaluronic acid production, acid agglutination and mouse pathogenicity.

TABLE 1

PROPERTIES OF *PAST. SEPTICA* STRAINS FROM VARIOUS SOURCES

Property examined	*Source*			
	Ox (5)	*Pig* (28)	*Dog* (39)	*Cat* (31)
Visible capsule	3 —	24 (83%)	5 (13%)	4 (13%)
Mucoid colonies	1 —	24 (83%)	0 (0%)	0 (0%)
Iridescence	4 —	24 (83%)	3 (8%)	0 (0%)
Hyaluronic acid	1 —	25 (89%)	1 (3%)	0 (0%)
Acid-agglutinability	1 —	3 (11%)	37 (95%)	30 (97%)
Mouse pathogenicity:				
++	4 —	26 (93%)	5 (13%)	24 (78%)
+	1 —	1 (4%)	18 (46%)	7 (23%)
—	0 —	1 (4%)	16 (41%)	0 (0%)

Note: Hyaluronic acid production. For the purpose of the Table, only strains giving an opacity equal to or greater than that of Brown's tube 1 following the mixing of saline culture extract with acid serum reagent are counted as positive.

Capsule

As shown in Table 1, smears stained by Jasmin's method showed zones interpreted as capsule or envelope in 36 of the 103 strains examined. Most positive strains were from pigs. It is unlikely that the zones were artefacts formed by shrinkage during drying, fixing or staining since many strains were negative, despite repeated tests. As controls, smears of a faecal streptococcus, a strain of *Staphylococcus albus* and a non-capsulated strain of *Escherichia coli* were stained by the same method; no zones were seen.

Colony Formation

When grown for 24 hours at 37°C on blood agar nearly all pig strains produced large, transparent, mucoid colonies up to 1·8 mm. diameter, which tended to become confluent; one bovine strain gave similar colonies (Table 1). The remaining strains (mainly from dogs and cats) produced colonies which differed little from one another; they were up to 1·2 mm. diameter, greyish, translucent, smooth and undifferentiated, and the blood was slightly browned. All strains had a faint but highly characteristic smell when grown on blood agar; this smell, once learnt, is of distinct value in the rapid screening of suspected *Past. septica* strains.

Non-mucoid strains in which a capsule was demonstrated

tended to form colonies slightly softer and more opaque than non-capsulated forms. Many strains from dogs and a few from cats agglutinated spontaneously in saline, some very strongly; their colonies, though not rough, were sometimes rather small. Saline-agglutinable strains usually produced a floccular or granular deposit in broth cultures; the rest gave an even turbidity.

Iridescence

Iridescence appeared to be closely linked with capsule or envelope production and 31 strains, mainly from pigs and cattle, produced iridescent growth on serum agar. Only three strains from dogs and none from cats were iridescent (Table 1). Viewed by obliquely transmitted sunlight the growth of iridescent strains appeared blue or violet, less often yellowish-green or pink. The colour could usually be altered by changing the angle of incidence of the light. No fluorescence was seen when cultures of iridescent and other strains were examined in the dark under a powerful ultraviolet light source (six inches from a 250 watt compact source mercury vapour lamp* filtered through an Ilford 828 glass filter).

Colony iridescence in *Past. septica* was first noted by de Kruif (1921) who referred to it as fluorescence and was followed in this respect by later writers including Webster and Hughes (1929), Cornelius (1931), Ochi (1933) and Elberg and Cheng Lee (1950). Fluorescence, however, is the property of certain materials of absorbing light of a specific frequency-range, in most cases ultraviolet, and re-emitting part of the absorbed energy as light of a greater wavelength. The colour effects given by *Past. septica* cannot be due to fluorescence since they are obtainable only by transmitted light, the colours can be altered by changing the angle of illumination and the phenomenon does not occur when filtered ultraviolet light is used. For these reasons it is better to use the term iridescence, which merely implies a play of changing colours.

It seems likely that the colour effects described are diffraction phenomena. The striking regularity of the arrangement of individual organisms in smears of capsulated or envelope-producing strains of *Past. septica* has already been mentioned. In the growth on solid media this regular mosaic of opaque coccobacilli and transparent capsules might well act as a series of diffraction gratings, and colour spectra could result if white light were passed through such a system. Moreover, assuming the individual organism to be 1·0 μ wide and almost spherical, and the capsule to be 0·5 μ thick, the grating pattern would be repeated every 2·0 μ or 12,700 times per inch, approximating fairly closely to the accepted value of about 14,000 per inch for optimum performance in diffraction gratings.

Hyaluronic Acid Production

Although the method used was only roughly quantitative, the

* General Electric Company.

results showed that mucoid cultures contained more hyaluronic acid than non-mucoid ones. The mean of all results from pig strains, which were mostly mucoid, was 4·8. This figure is the mean turbidity on Brown's scale of saline culture extracts after the addition of acid serum reagent. In contrast, the mean results for strains from cattle, dogs and cats were 0·53, 0·24 and 0·097 respectively. Of the strains from pigs, 25 of the 28 gave results of 1·0 or more, as against only one bovine, one canine and no feline strain (Table 1).

Acid-agglutination

The results are summarized in Table 1 and further details in Table 2. In general, pig and ox strains were inagglutinable by acid, perhaps as a result of capsule or hyaluronic acid production, while most dog and cat strains were agglutinated over the pH range 2·5 to 4·5.

TABLE 2

AGGLUTINATION OF *PAST. SEPTICA* STRAINS BY ACID

Agglutination pattern					*Strains agglutinated*			
pH 2·5	3·5	4·0	4·5	5·5	*Ox*	*Pig*	*Dog*	*Cat*
—	—	—	—	—	4	25	2	1
+	—	—	—	—	0	0	2	0
+	+	—	—	—	0	1	0	0
+	+	+	+	—	1	2	31	30
+	+	+	+	+	0	0	3	0
—	—	+	+	—	0	0	1	0

Mouse Pathogenicity

Most of the strains from cattle, pigs and cats were highly pathogenic for mice, whereas strains from dogs were generally of low pathogenicity or non-pathogenic (Table 1). Most deaths occurred within 48 hours of inoculation; the lesions were those of a septicaemia, with enlarged spleen, enlarged and congested lymph nodes, yellowish, mucoid material in the intestines, and a pale or clay-coloured liver. Mice dying later sometimes showed pneumonic areas in the lungs and a suppurative pericarditis.

There was some association between capsule or envelope production and mouse pathogenicity, as shown in Table 3. Strains in which capsule or envelope could be demonstrated were almost always highly mouse pathogenic, but 25 of the apparently non-capsulated strains, mainly from cats, were also highly pathogenic, suggesting that although capsule or envelope may be capable of conferring mouse pathogenicity, they are probably not the only factors involved.

Fermentation Reactions

The fermentation reactions of the 103 strains examined are

summarized in Table 4. Xylose, sorbitol and mannitol gave similar results, which closely parallelled mouse pathogenicity; these substances were usually fermented by strains from cattle and pigs but rarely by those from dogs (Table 4). Maltose, trehalose and dextrin on the other hand were fermented predominantly by dog strains while cat strains appeared to occupy an intermediate position.

TABLE 3

MOUSE PATHOGENCITY OF CAPSULATED AND NON-CAPSULATED STRAINS OF *PAST. SEPTICA*

Capsule or envelope	*Mouse pathogenicity*		
	++	+	—
+	34 strains	3 strains	2 strains
—	25 „	24 „	15 „

TABLE 4

FERMENTATION REACTIONS OF *PAST. SEPTICA* STRAINS FROM VARIOUS SOURCES

Substance fermented		*Number of strains giving fermentation*			
		Ox (5)	*Pig* (28)	*Dog* (39)	*Cat* (31)
Pentoses	Xylose	4	27	9	17
	Arabinose	1	1	2	0
	Rhamnose	0	1	3	0
Hexoses	Glucose	5	28	39	31
	Mannose	5	26	33	30
	Galactose	5	28	39	28
	Fructose	5	28	39	30
Disaccharides	Sucrose	5	28	39	31
	Maltose	0	1	17	9
	Lactose	0	1	4	6
	Trehalose	0	11	26	13
Trisaccharide	Raffinose	0	4	8	7
Polysaccharides	Starch	0	1	2	3
	Dextrin	0	0	15	4
	Glycogen	0	1	0	4
	Inulin	0	1	0	0
Alcohols	Sorbitol	5	27	7	17
	Mannitol	5	28	6	24
	Dulcitol	0	0	1	0
	Glycerol	4	16	4	23
	Erythritol	0	0	0	0
	Inositol	0	1	0	0

Note: Gas production was tested for in glucose and sucrose only; all strains were negative.

Differences between Strains from Healthy and Diseased Animals

Detailed examination of the results showed on the whole little difference between strains from healthy and diseased animals of the same species. No difference was found between strains from healthy dogs and dogs suffering from minor ailments such as tonsillitis. Strains from these two groups, however, fermented maltose, trehalose and dextrin more frequently than those from serious disease in dogs (pneumonia, septicaemic conditions) while the latter were more active against xylose, sorbitol and mannitol (see Table 5). Too much weight should not be attached to these results since relatively few strains from serious disease were available.

Cultures from serious disease in dogs tended to be strongly mouse pathogenic; three were ++, three + and none —. Only two of the 33 cultures from healthy dogs and minor ailments in dogs were ++ mouse pathogenic, but 15 were + and 16 non-pathogenic. Strains from healthy cats fermented maltose slightly more often than those from diseased cats. Since nearly all strains from cattle and pigs were from diseased animals, no comparisons were possible within these species.

TABLE 5

FERMENTATION REACTIONS OF *PAST. SEPTICA* STRAINS FROM HEALTHY AND DISEASED DOGS

Substance fermented	*Number of strains positive*	
	From healthy dogs and dogs with minor ailments (33)	*From serious disease (6)*
Maltose	15	1
Trehalose	21	2
Dextrin	15	0
Xylose	4	3
Sorbitol	4	3
Mannitol	3	3

DISCUSSION

Pasteurella septica has been isolated from a wide variety of mammals and birds, and although the species has long been known to be heterogeneous, early studies revealed little difference between strains from different host species. Avian strains, however, are generally agreed to ferment arabinose, a substance rarely attacked by mammalian strains, and also to ferment mannitol but not xylose; the original observation of this fact was made by Khalifa (1934) and has been repeatedly confirmed. The failure of most work prior to 1950 to detect other differences between cultures from different animals led to the abandonment of specific names indicating the

host, such as *equiseptica*, *boviseptica* and *oviseptica*, and their replacement by the group name *Pasteurella septica* (Topley and Wilson, 1931); the term *Pasteurella multocida* was later suggested (Rosenbusch and Merchant, 1939) for similar reasons. The present results contain several examples of real or apparent association between in vitro characteristics and host species: thus hyaluronic acid production, giving rise to mucoid colonies, appeared an almost constant characteristic of pig strains, but it must be remembered that most of these came from chronic pneumonia and further work might greatly alter the picture; a high mouse pathogenicity in the absence of demonstrable capsule or iridescence was characteristic of cat strains in the present study; maltose, trehalose and dextrin fermentation was characteristic of dog strains and fermentation of xylose, sorbitol and mannitol of strains from farm animals. It is not suggested that these differences are sufficient grounds for resurrecting the old specific names to subdivide what is in many other respects a uniform and satisfactory species.

Certain of the differences described are no doubt due to the smooth-rough variation which has already been fully described for the species (Webster and Burn, 1926; de Kruif, 1921, 1922; Anderson, Coombes and Mallick, 1929; Hughes, 1930). Carter (1957) proposed a satisfactory simplification of previous classifications to include three types only, mucoid, smooth (which may or may not be capsulated) and rough. The present results agree well with this classification; pig strains were predominantly mucoid, strains from cattle were predominantly smooth and capsulated, those from cats were mainly smooth and non-capsulated and those from dogs, though varied, were mainly rough. It is of interest that smooth strains predominated in healthy and diseased cats alike.

The presence of a visible capsule in a strain of *Past. septica* can apparently indicate production of either capsular polysaccharide or hyaluronic acid; indeed a mucoid strain might well produce both. The results suggest that iridescence and acid-inagglutinability are usually due to the presence of one or both of these substances. The presence of either substance also seems to confer a high mouse pathogenicity on the strain but is probably not the only factor concerned, for the majority of cat strains, though non-capsulated, were highly mouse pathogenic.

As previously noted (Smith, 1955) disease due to *Past. septica* is rare in dogs although the organism is widely present in the nose and tonsils of healthy dogs. It seems likely that the particular type of rough or partly rough, maltose-fermenting strain found in dogs is one well adapted to a benignly parasitic existence and not predisposed to give rise to illness. The differences between dog and cat strains, particularly the high mouse pathogenicity and smooth nature of cat strains as opposed to the low pathogenicity and rough nature of those from dogs, might also explain why local infections

due to *Past. septica* are rare in human beings bitten by dogs, but are commoner after cat bites.

CONCLUSIONS

Pasteurella septica strains from a variety of animal sources showed certain cultural and biochemical differences.

Strains from lung lesions in pigs usually produced mucoid colonies containing a considerable quantity of hyaluronic acid. They were visibly capsulated, iridescent, acid-inagglutinable and highly mouse pathogenic, fermenting xylose, sorbitol, mannitol and sometimes trehalose but rarely maltose or dextrin.

Strains from cattle generally produced non-mucoid colonies containing little or no hyaluronic acid; in other respects they resembled those from pigs.

Dog strains produced non-mucoid colonies containing little or no hyaluronic acid. They were usually non-capsulated, non-iridescent, acid-agglutinable, saline-agglutinable and of little or no mouse pathogenicity; trehalose and sometimes maltose and dextrin were characteristically fermented.

Cat strains were usually mannitol positive, and though non-iridescent and apparently non-capsulated, were generally highly mouse pathogenic.

Minor differences in fermentative ability were observed between strains from healthy and diseased animals of the same species.

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THE INTER-RELATIONSHIP BETWEEN TUBERCULOSIS AND BOVINE FARCY

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INTRODUCTION

Bovine farcy has been recognized in Sudan for many years, but it is only very recently that the significance of its relation to tuberculosis and tuberculin sensitivity has been appreciated. The study of the disease in this country is of much importance as it may apparently sensitize animals to tuberculin and so cause a major complication in interpreting the results of tuberculin testing.

Henrici and Gardner (1921) reported that acid-fast actinomycetes are closely related to tubercle bacilli in morphological and cultural characters, and in pathogenicity and serological reactions. They stated that actinomycetes produce infections that resemble tuberculosis with which they may be confused even on microscopic examination. They added that the natural disease may resemble tuberculosis and observed that guinea pigs inoculated with acid-fast actinomycetes gave positive tuberculin reactions. Daubney (1927) describing the histopathology of actinomycotic infection of the udder stated that the lesions have a striking resemblance to those of bovine tuberculosis and that the whole process is in the closest agreement with M'Fadyean's classical description of the histological changes in tuberculous bovine mastitis. He concluded that a relationship appears to exist between *Actinomyces farcinicus* and *Mycobact. tuberculosis* and that the diseases produced have also many points in common. Goyal (1937) prepared extracts from different cultures of Nocardia species in a manner comparable to that used in the preparation of tuberculin. Animals sensitized to Nocardia extracts were sensitive to tuberculin and vice versa. Serological studies confirmed the conclusion reached on the basis of allergy tests; a common antigen was demonstrated for tubercle bacilli and Nocardia. These results led to the conclusion that there is a definite relationship between actinomycetes and mycobacteria. Drake and Henrici (1943) showed that rabbits and guinea pigs infected with *N. asteroides* give a specific delayed reaction to a protein and a polysaccharide fraction of *N. asteroides* and to extracts of the powdered organisms, and that these extracts do not cause reactions in tuberculous animals. Hagan (1946) stated that some cattle affected with bovine farcy react to tuberculin. Awad and Karib (1957) studied bovine farcy among cattle in Sudan and reported that two distinct types of the disease occur, a chronic purulo-granulomatous inflammation of the subcutis involving the lymph nodes and vessels, and an internal type the pulmonary form of which closely resembles tuberculosis.

FIELD CASES

Appearance of Similar Lesions in Tuberculosis and Farcy

Eight cases of apparent tuberculosis were encountered at

Malakal slaughter house. The lesions were mainly in the lungs and the thoracic lymph nodes, and in the prescapular and retro-pharyngeal lymph nodes. In one animal the pleura was heavily infected with the usual appearance of "grapes" disease. Typical acid-fast actinomyces were demonstrated in three animals including that with the infected pleura. Material from each animal was separately inoculated subcutaneously into a guinea pig. Of the eight inoculated animals three died within 5 to 9 weeks after inoculation showing caseation of the precrural and inguinal lymph nodes as well as necrotic foci in the spleens and livers. Typical acid-fast tubercle bacilli were found in smears from the lesions. The remaining five guinea pigs developed localised abscesses at the sites of inoculation from which creamy pus was discharging; typical acid-fast actinomyces were demonstrated. When killed four months after inoculation no internal lesions could be detected in any of the guinea pigs.

Tuberculosis and Farcy in the same Animal

A post-mortem examination of a 7-year old bull at Khartoum revealed a large subcutaneous abscess at the base of the right ear and caseated lesions in the bronchial and mediastinal lymph nodes. A few acid-fast bacilli and branching filaments were found in smears from the nodes. Material from the lymph nodes was inoculated subcutaneously into a guinea pig. Three weeks later *A. farcinicus* was demonstrated in smears from the local abscess which had developed at the site of inoculation. On post-mortem examination seven weeks after inoculation, the precrural and inguinal lymph nodes were found to contain caseous material and there were necrotic foci in the spleen and liver. Smears from the lesions showed acid-fast bacilli morphologically typical of tubercle bacilli. Pus from the precrural lymph node was inoculated subcutaneously into another guinea pig; at post-mortem eight weeks later, lesions containing acid-fast bacilli were demonstrated in the precrural, inguinal lymph nodes, spleen and liver.

Samples of liver and prescapular lymph nodes of a bull suspected of being affected with bovine farcy were received from El-Obied. Smears from the liver lesions, which macroscopically were similar to those in the lymph nodes, revealed acid-fast bacilli; but smears from the lymph nodes showed typical acid-fast actinomyces. Material from the liver and lymph nodes were separately inoculated subcutaneously into two guinea pigs. The liver produced typical tuberculous lesions in the guinea pig and acid-fast bacilli were demonstrated; material from the lymph nodes, however, produced only a localised abscess at the site of inoculation from which typical acid-fast actinomyces were demonstrated.

Tuberculin Reactions in Farcy

Ninety cows of the Malakal dairy herd were subjected to an

intradermal test with mammalian tuberculin. Eighteen reacted and were subjected to a thorough post-mortem examination the results of which are shown in Table 1, which shows that three of the eighteen reactors proved to be affected with bovine farcy and not tuberculosis.

TABLE 1

RESULTS OF POST-MORTEM EXAMINATIONS

No.	*Visible P.M.* lesions*	*Culture†*	*Subcut. inoculation of guinea pigs*
1	+	+	T.B. lesions
5	+	—	farcy lesions
8	+	+	T.B. lesions
9	+	—	T.B. lesions
17	+	+	T.B. lesions
25	—	—	T.B. lesions
33	+	+	T.B. lesions
35	+	—	farcy lesions
36	+	+	T.B. lesions
37	+	+	T.B. lesions
38	+	+	T.B. lesions
53	+	—	farcy lesions
55	+	+	T.B. lesions
59	+	+	T.B. lesions
71	+	+	T.B. lesions
77	+	+	T.B. lesions
79	—	+	T.B. lesions
83	—	—	T.B. lesions

*The lesions were mainly confined to the respiratory system.

†Growth of *Myco. tuberculosis* on Lōwenstein's medium (Jensen, 1953).

EXPERIMENTAL FINDINGS

Guinea Pigs

Twelve animals were divided into three groups of four animals each. The first group was inoculated subcutaneously and the second intraperitoneally with material from natural cases heavily infected with *A. farcinicus*. The third group was inoculated intraperitoneally with a 34-days old culture on potato agar of *A. farcinicus*. Four tuberculous guinea pigs were included as controls. Three to four weeks after inoculation all the animals were subjected to the single intradermal test with 0·1 ml. mammalian tuberculin. Three animals from the first, two from the second and two from the third group, and all the controls reacted positively.

Cattle

Twelve calves about 3 to 4 months old were found negative to the single intradermal comparative test. Two calves were kept as controls. Ten were experimentally infected with *A. farcinicus* as follows. Six calves received material from natural cases (192 and 193

subcutaneously, 197 and 236 intradermally, 200 and 202 by inhalation). Four calves received a 40-days old culture of *A. farcinicus* on potato agar (191 and 271 intradermally, 198 and 201 subcutaneously). Sixty-four days after inoculation the twelve animals were divided into two groups of five calves and a control each. The first group was tested intradermally with mammalian tuberculin (Table 2) and the second group with mammalian and avian tuberculins. In the second group (Table 3) four out of five calves reacted positively to both tuberculins, but the mammalian increase did not exceed the avian by more than 2 mm.

All the ten animals were retested 72 days later using the comparative test. The results (Table 4) show that according to the accepted interpretation of the test all the calves should be considered negative for bovine tuberculosis.

TABLE 2
SINGLE INTRADERMAL TUBERCULIN TEST

No.	*Measurements (mm) Mammalian tuberculin*		*Difference (mm)*	*Result*
	1st Reading	*2nd Reading*		
193	4	9	5	+
197	5	12	7	+
200	5	8	3	±
191	6	11	5	+
198	4	10	6	+
Control	5	6	1	—

TABLE 3
SINGLE INTRADERMAL COMPARATIVE TUBERCULIN TEST

No.	*Measurements (mm)*			
	Avian tuberculin		*Mammalian tuberculin*	
	1st Reading	*2nd Reading*	*1st Reading*	*2nd Reading*
192	3	6	3	8
236	4	10	5	12
202	5	10	5	11
271	4	10	4	11
201	4	9	5	12
Control	4	4	4	4

TABLE 4

No.	Measurements (mm.)			
	Avian tuberculin		Mammalian tuberculin	
	1st Reading	2nd Reading	1st Reading	2nd Reading
193	4	4	4	5
197	4	7	5	8
200	4	4	5	5
191	6	9	6	6
198	4	10	4	6
192	3	10	3	6
236	4	6	5	6
202	5	12	5	6
271	4	8	4	5
201	4	5	5	5

DISCUSSION

Bovine farcy is common in Sudan but the position in relation to tuberculosis is not yet clear. The disease is not so rare as is generally believed, especially in the South (Awad, 1957). Awad and Karib (1957) have shown that, in many cases, the internal form of bovine farcy can be confused with bovine tuberculosis. In the Sudan and in countries which import Sudanese cattle, this similarity has led to the condemnation of carcasses as tuberculous. The confusion between the two diseases may even include microscopical examination, especially where, because of advanced caseation or calcification, the mycelia in bovine farcy have broken down into forms resembling tubercle bacilli. During the present investigation it was found that microscopical examination alone is not sufficient to establish a definite diagnosis; moreover mixed infections occur which add to the problem of differentiation by microscopical examination. This difficulty is mentioned by Wilson and Miles (1948) who reported that microscopical examination alone cannot distinguish between tubercle bacilli and other acid-fast organisms, and by Kolmer, Spaulding and Robinson (1957) who advised that specimens suspected of containing actinomyces should be routinely examined for tubercle bacilli, a procedure adopted in the present investigation. It is only possible to differentiate with certainty between farcy and tuberculosis by guinea pig inoculation. If suspected material containing *A. farcinicus* is inoculated subcutaneously into a guinea

pig, a localised abscess develops within four weeks at the site of inoculation and discharges a creamy pus containing typical acid-fast actinomyces; extension of infection never occurs.

If the material contains tubercle bacilli, the regional lymph node enlarges and contains caseous material, infection extends to the spleen and liver and the animal dies within six to eight weeks. If the suspected material contains both *A. farcinicus* and tubercle bacilli, a localised abscess develops at the site of inoculation and from it actinomyces can be demonstrated, while the tubercle bacilli invade the body and form lesions in the regional lymph node, spleen and liver in which tubercle bacilli can be found. During the tuberculin testing campaign at Malakal the existence of farcy caused positive reactions to mammalian tuberculin in a number of cows which at autopsy were found to be free from tuberculosis.

The experiments with guinea pigs show that *A. farcinicus* may sensitize animals to either avian or mammalian tuberculin or to both. Affected animals respond irregularly to tuberculin and the absence of a reaction does not eliminate the possibility of the existence of farcy. The experiments with cattle show that the response of farcy animals to tuberculin is not constant or permanent and retesting of reactors may permit their retention in the herd. Where farcy is known to exist, the single intradermal comparative tuberculin test is preferable and reacting animals should be retested after sixty days.

CONCLUSIONS

Bovine farcy has been recognised in Sudan for many years, but it is only very recently that the significance of its relation to tuberculosis and tuberculin sensitivity has been appreciated.

Macroscopical and microscopical examinations are not sufficient to distinguish farcy from tuberculosis. The best method is guinea pig inoculation.

Experiments in calves and guinea pigs show that *A. farcinicus* may sensitize animals to avian or mammalian tuberculin or both and may thus confuse the interpretation of tuberculin tests. Affected animals respond irregularly to tuberculin and the absence of a reaction does not eliminate the possibility of the presence of bovine farcy.

The single intradermal comparative tuberculin test should be applied wherever bovine farcy is known to exist, and reacting animals should be retested after sixty days.

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THE EXPERIMENTAL INFECTION OF CATTLE WITH *MYCOBACTERIUM JOHNEI*

I. Calves Inoculated Intravenously

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INTRODUCTION

In an attempt to find a repeatable method of inducing Johne's disease in cattle, Taylor (1953) inoculated calves intravenously with 100 mg. of a mixture of five recently isolated strains of *Mycobacterium johnei*. Clinical Johne's disease was produced in three out of four calves inoculated at three months of age or less, but neither symptoms nor lesions of the disease were produced in two calves inoculated at six months of age. The experiments to be described in this and succeeding papers were initiated to determine whether or not Taylor's results could be repeated and to investigate further his observations on apparent age resistance.

MATERIALS AND METHODS

The organism used was a strain of *M. johnei* which had been recovered from one of the clinical cases of Johne's disease set up by Taylor (1953). The isolation was made on Taylor's modification of Finlayson's medium (Taylor, 1950) and the bacilli injected were from an eighth sub-culture on the same medium. The bacilli were non-infective for guinea-pigs and rabbits and would not grow without the phlei-factor. Inoculations were made into the jugular vein, the organisms being suspended in Ringer's solution at a concentration of 10 mg. wet weight of bacilli per 1 ml. solution.

Experimental animals. Heifer calves born on this Field Station and predominantly of the Ayrshire breed were used. Only strong healthy calves which showed no reaction to either avian or mammalian tuberculin were selected.

Maintenance. The experimental animals were at all times kept in an isolation unit and had no access to the open. They were allowed exercise in an enclosed yard, the floor of which was of concrete. Calves were given their mother's colostrum for the first three days, after which they were fed on bulk milk, milk substitute, hay and calf-nuts*, according to the table. After the rearing period, diet varied according to the season of the year, grass or lucerne being supplied in summer and kale or mangolds in winter.

Adult cattle received a basic ration of 12 lb. to 16 lb. of hay plus 40 lb. of cut grass in summer, or 30 lb. of kale, or 25 lb. of mangolds in winter. In addition those cows in-milk received 4 lb. of a mixture of equal parts of oats and "Grain Balancer" for the first 2 gallons of milk and a further 4 lb. "Red Label" milk nuts (British Oil and Cake Mills) for each

* (British Oil & Cake Mills).

additional gallon. Water was always available *ad. lib.* Mature cattle were milked in the usual manner and the aim was to have them calving once a year and so to subject them to the principal stresses normal to the cow in the commercial dairy herd.

Week	*Whole milk per day in pints*	*Milk substitute per day in pints*	*Calf nuts*	*Meadow hay*	*Drinking water*
1	6	—	—	Available *ad lib*	—
2	8	—	—	,,	—
3	8	—	—	,,	—
4	5	3	Available *ad lib*	,,	—
5	3	5	,,	,,	—
6	1	7	,,	,,	—
7	—	8	,,	,,	—
8	—	8	,,	,,	Available *ad lib*
9	—	6	,,	,,	,,
10	—	6	,,	,,	,,
11	—	4	,,	,,	,,
12	—	4	,,	,,	,,

Technique for recovering M. johnei. Cultivation of the organism from faeces or post-mortem material was by either the antiformin or the oxalic acid method described by Taylor (1950). The oxalic acid method was used for all "clean" materials, e.g. lymph nodes, liver, spleen, etc: whereas the antiformin method was reserved for heavily contaminated material and organs opening to the surface of the body, e.g. faeces, intestine, bladder, lung, etc. Inoculations were made on slopes of Finlayson's inspissated egg as modified by Taylor (1950), five slopes being used for each sample. Slopes were incubated at 38°C. They were examined after 48 hours for contaminants and then at monthly intervals for three months after which they were discarded.

Method of infection. Six one-month-old heifer calves were inoculated intravenously with 100 mg. wet weight of *M. johnei*. They were then reared as a group and mated, calved, and milked in the normal manner. Faeces samples were collected at monthly intervals and examined culturally for the presence of *M. johnei*. At post-mortem examination the animals were

examined macroscopically, microscopically, and culturally for Johne's disease or the presence of *M. johnei*.

RESULTS

No immediate effect of the inoculation was observed. The calves behaved and fed in the normal manner. At a later stage, however, it was noticed that some of the calves inoculated intravenously did not grow as well as uninoculated calves of similar breeding kept under the same conditions and that, as adults, they were stunted (Fig. 1).

Excretion of M. johnei

The results of the cultural examinations of the faeces samples are shown in Table 1. It will be seen that during the first year all six calves were excreting *M. johnei* more or less consistently. Three of them (Nos. 2, 3 and 5) were killed because of Johne's disease within 15 months of receiving the inoculum. Of the remaining three, No. 1 excreted the organism consistently for 3 years and was eventually killed as a clinical case of Johne's disease at $3\frac{1}{2}$ years of age. Numbers 4 and 6 stopped excreting the organism after the 15th month, except for one isolated instance in each case. When they were eventually killed at 4 years of age they were showing no evidence of disease.

Clinical Observations and Post-mortem Findings

No. 1. This was one of the animals which was stunted in growth. Apart from this she remained apparently healthy until about 3 years old when she calved for the first time. Shortly after calving she showed evidence of diarrhoea. She milked for only 109 days giving a total yield of 1,064 lb. and was destroyed as a clinical case of Johne's disease 5 months after calving. Post-mortem examination revealed the most strikingly affected intestine of any of the group. The bowel mucosa was markedly thickened and corrugated from end to end. The corrugations were particularly tight but there was no haemorrhage (Fig. 2). This intestine was four times the bulk of intestines showing the usual lesions of Johne's disease.

Histological examination of the intestine showed advanced lesions of Johne's disease and very numerous acid-fast bacilli in the duodenum, ileum, caecum and rectum. The results of the cultural examination are shown in Table 2.

No. 2. This heifer began to show signs of intermittent diarrhoea 8 months after inoculation. By the eleventh month she was scouring profusely and had lost a lot of flesh. She was killed as a clinical case of Johne's disease. At post-mortem examination the ileum and caecum showed the usual thickened and corrugated mucous membrane and histological examination of sections of the intestine confirmed the diagnosis of Johne's disease. The results of the cultural examination are shown in Table 2.

No. 3. The first attack of diarrhoea occurred 7 months after inoculation. The heifer failed to grow normally and when she was killed 15 months

TABLE 1

RESULTS OF MONTHLY CULTURAL EXAMINATIONS OF FAECES

Samples from calves experimentally inoculated at 1 month of age with 100 mg. *M. johnei* by the intravenous route

| *No. of calf* | *Months after infection* | | |

after infection she was a typical case of Johne's disease. At post-mortem examination the intestine was found to be thickened and corrugated from duodenum to rectum. Lesions of Johne's disease, confirmed histologically, were seen in all sections of the intestine. The results of the cultural examination are shown in Table 2.

TABLE 2

RESULTS OF CULTURAL EXAMINATIONS OF TISSUES

Various organs from cattle inoculated intravenously with 100 mg. *M. johnei* at one month of age

Site cultured	*Calf No.*					
	1	*2*	*3*	*4*	*5*	*6*
Duodenum	+	+	+	—	+	—
Ileum	+	+	+	—	+	—
Caecum	+	+	+	—	+	—
Rectum	+	+	+	—	+	—
Uterus	+			—		—
Ovary	+			—		—
Udder	+			—		—
Lung		+	+	—	C	—
Kidney		+	+	—	+	
Liver		+	+	—	+	
Spleen		+	+	—	+	
Lymph Nodes:—						
Submaxillary		+	+	—	+	—
Retropharyngeal		+	+	+	+	—
Prescapular		+	+	—	+	—
Popliteal		+	+	—	+	—
Iliac		+	+	—	+	—
Hepatic				+		
Mediastinal		+	+	—	+	—
Ileo-caecal	+	+	—	—	+	+
Mesenteric	+	+	—	+	+	—
Supramammary		+	+	—	+	—

+ = One or more colonies *M. johnei* recovered.
— = No *M. johnei* recovered.
C = All five slopes contaminated.

No. 4. This was one of two animals which, on the evidence of cultural examinations of the faeces, appeared to throw off the infection since only one isolation of the organism was made after the fifteenth month. She put on a considerable amount of weight and was difficult to get in calf. She calved for the first time at $3\frac{1}{2}$ years of age and milked for only 131 days yielding 1,556 lb. of milk. She was killed 4 years after infection when she was still apparently healthy. Post-mortem examination failed to reveal any macroscopic evidence of Johne's disease. Histological examination of sections of the duodenum, ileum, caecum and rectum was also negative. *M. johnei* was, however, recovered in pure culture from three lymphatic nodes associated with the intestinal tract (Table 2).

No. 5. The first signs of diarrhoea were seen 10 months after infection. Diarrhoea was intermittent for a time but by the fifteenth month it was more or less continuous and the heifer was killed as a clinical case. Post-mortem examination showed typical lesions of Johne's disease with thickening and corrugation of the intestinal mucous membrane extending from the duodenum to the rectum. Sections made from various sites in the bowel confirmed the diagnosis. The results of the cultural examination are shown in Table 2.

No. 6. This animal grew well and from the cultural examination of her faeces she also, like No. 4, appeared to throw off the infection. She was difficult to get in calf and was served or inseminated on 10 occasions before she conceived. She was $3\frac{1}{2}$ years of age when she calved and she milked for 259 days giving 4,529 lb. of milk. She was killed when nearly $4\frac{1}{2}$ years of age and still apparently normal. At post-mortem both macroscopic and histological examinations of the intestine failed to reveal any evidence of Johne's disease. *M. johnei* was, however, recovered from the caecum and ileo-caecal lymph node (Table 2).

DISCUSSION

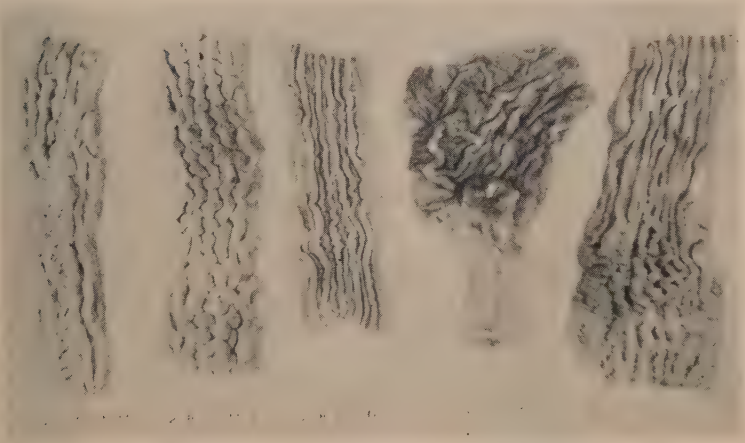
The experiment confirms that Johne's disease in its typical clinical form can be produced in cattle by the inoculation of *M. johnei* into the blood stream of calves one month old. The incubation period was shorter than that usually found in naturally affected cases for three of the four clinical cases had broken down by the fifteenth month after infection, whereas the average age for death in the field is $2\frac{1}{2}$ to 4 years. This early appearance of the disease may explain the stunting of growth seen in some of the experimental animals but which is not a feature of the naturally occurring disease. At first it was thought that the stunting might be the result of lung damage due to massive invasion of the alveolar walls by bacilli from the venous circulation. It is, however, noteworthy that the two animals which did not show intestinal lesions post-mortem were of normal size although they also were inoculated intravenously. It seems likely that in some cases the intestine is attacked soon after infection and its usefulness for digestion and absorption is impaired at a time when growth rate is rapid.

The two cows found to be normal when killed at about 4 years of age had been excreting bacilli more or less regularly for fifteen months after the infective dose had been given: thereafter the bacilli

Fig. 1

Four cows (half sisters, related through the bull) photographed when 3 years of age and reared together since birth. The second and fourth from the left were infected when one month of age with *M. johnei* by the intravenous route.

Fig. 2



Portions of the intestine of the 3½ year old cow No. 1 illustrating the marked lesions of Johne's disease produced by the intravenous inoculation of *M. johnei* when she was 1 month old.

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disappeared from the faeces. As it is unlikely that excretion would have been so regular in the absence of an intestinal lesion it is assumed that these two cattle had intestinal lesions which regressed after they were 15 months old. The fact that the organism was recovered from both these cattle at post-mortem, makes reinvasion of the intestine always a possibility.

As regards the pathogenesis of Johne's disease two conditions have to be considered, the first in which *M. johnei* is present in the tissues, principally the lymph nodes, without causing any apparent disease, and the second in which the mucous membrane of the intestine is invaded and altered. The term "Johne's disease" should be used exclusively to describe the latter.

CONCLUSIONS

Four of six calves inoculated intravenously when one month old with *Mycobacterium johnei* developed clinical Johne's disease.

The incubation period was about one half of that usually found in field cases.

The growth of animals which became clinical cases tended to be checked.

Two animals which did not become clinical cases were still harbouring the organism 4 to $4\frac{1}{2}$ years after infection.

It is suggested that the term "Johne's disease" should only be used in cases where there is an intestinal lesion.

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THE DEVELOPMENT AND SURVIVAL OF THE PREPARASITIC STAGES OF *NEMATODIRUS* SPP. ON PASTURE HERBAGE

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INTRODUCTION

Nematodiriasis has, in the last few years, been recognised as a serious disease of young lambs, the control of which is of paramount importance to the sheep farmer. In the absence of an efficient anthelmintic for prophylactic and therapeutic use, control depends upon husbandry methods which demand a thorough knowledge of the epidemiology of the disease and of the biomonics of the preparasitic stages on the pasture herbage. The experiment described in this paper was therefore devised to yield information on the development and survival of the preparasitic stages on the pasture herbage.

MATERIALS AND METHODS

An area of ground, eight feet square surrounded by a continuous concrete kerb and contained within an enclosure covered with bird-proof wire netting, was sown in the autumn of 1954 with a ryegrass-clover mixture. In the spring of 1955 when the sward had become established galvanised iron sheets were driven into the turf in such a way as to divide it up into squares each of one square foot in area. The metal sheets projected some four inches above the surface of the soil so as to prevent the accidental transference of material from plot to plot. In order to study the effects of the weather on the development and survival of the preparasitic stages of *Nematodirus* spp. it was intended that sheep faeces containing eggs should be placed serially on the plots once a month over a period of two years. This intention was, however, frustrated on several occasions by the absence of faeces containing a sufficient number of eggs of *Nematodirus* spp. to make observations practicable. On each occasion, except the first, faeces were placed on two plots, one of these receiving 100 pellets of freshly passed sheep faeces and the other a pultaceous mass prepared by mixing 100 pellets of faeces with a suitable quantity of water. For purposes of identification the plots were numbered from 1 to 27, plot 1 being the first to receive faecal material on 13th April, 1955 and plot 27 the last on 12th June, 1956.

Material from the plots was examined at fortnightly intervals. One or two pellets, depending on size, or an equivalent portion of the pultaceous mass was collected and soaked in tap water in a two ounce spring-topped bottle overnight, being stored in the refrigerator to inhibit development beyond the stage reached at the time of collection. The material was then transferred to a cavity block along with a small quantity of the water in which soaking had taken place and was gently broken up with a small spatula. The resulting faecal suspension was passed through a screen supported over a bowl in which the fluid passing through the screen was

TABLE 1

RATE OF DEVELOPMENT AND TIME OF SURVIVAL OF FREE-LIVING STAGES OF *N. FILICOLLIS*

Date placed on plot	*Faecal pellets*			*Pultaceous faeces*		
	Third stage larvae present in eggs weeks	*First free larvae seen in faeces weeks*	*Free larvae last seen in faeces weeks*	*Third stage larvae present in eggs weeks*	*First free larvae seen in faeces weeks*	*Free larvae last seen in faeces weeks*
13.4.55	6	8	52	—	—	—
11.5.55	4	8	52	4	8	52
8.6.55	4	4	52	6	10	52
7.7.55	—	—	—	—	—	—
4.8.55	8	8	52	6	8	52
2.9.55	6	19	44	3	28	47
28.9.55	8	40	52	30	36	52
26.10.55	30	36	52	30	36	52
23.11.55	—	—	—	—	—	—
21.12.55	—	—	—	—	—	—
24.1.56	15	25	49	15	23	50
29.2.56	10	16	52	10	18	52
29.3.56	—	—	—	—	—	—
16.4.56	7	13	52	5	11	49
17.5.56	5	9	52	5	11	52
12.6.56	3	5	52	3	9	46

TABLE 2

RATE OF DEVELOPMENT AND TIME OF SURVIVAL OF FREE-LIVING STAGES OF *N. BATTUS*

Date placed on plot	*Faecal pellets*			*Pultaceous faeces*		
	Third stage larvae present in eggs weeks	*First free larvae seen in faeces weeks*	*Free larvae last seen in faeces weeks*	*Third stage larvae present in eggs weeks*	*First free larvae seen in faeces weeks*	*Free larvae last seen in faeces weeks*
13.4.55	30	47	52	—	—	—
11.5.55	22	46	52	24	46	52
8.6.55	22	48	48	22	46	46
7.7.55	—	—	—	—	—	—
4.8.55	30	Not seen	Not seen	43	43	47
2.9.55	10	30	42	10	32	52
28.9.55	40	44	52	38	46	52
26.10.55	30	40	50	34	Not seen	Not seen
23.11.55	—	—	—	—	—	—
21.12.55	—	—	—	—	—	—
24.1.56	27	38	38	23	36	36
29.2.56	22	28	52	20	29	52
29.3.56	—	—	—	—	—	—
16.4.56	13	17	52	11	16	52
17.5.56	9	15	41	9	33	49
12.6.56	8	11	47	8	9	47

caught. The débris remaining on the screen was washed with the water remaining from the original soaking. All the fluid caught in the bowl was then transferred to a Clayton Lane centrifuge tube and spun at 1,500 r.p.m. for two minutes. After decanting the supernatant fluid the tube was filled with a saturated solution of common salt and any eggs and larvae present recovered by the usual flotation technique. Examination of these eggs and larvae was made to determine the numbers of each present and the stage of development reached. Examination of material from any given plot ceased one year after the date when the faeces were placed on the plot.

Throughout the experiment temperature records were made by means of a thermograph housed in a standard Stevenson screen. Daily rainfall readings were also taken.

TABLE 3

RATE OF DEVELOPMENT OF EGGS OF *NEMATODIRUS* SPP. WHICH WERE PLACED ON GRASS DURING APRIL

Date of collection of faeces	*State of faeces*	N. filicollis						N. battus					
		No development	*Early segmentation*	*Morula stage*	*Tadpole stage*	*Third stage larvae*	*Free larvae*	*No development*	*Early segmentation*	*Morula stage*	*Tadpole stage*	*Third stage larvae*	*Free larvae*
11.5.55	Dry and hard			4	2			4					
25.5.55	Dry and hard					6							
8.6.55	Dry and hard					2	1						
23.6.55	Dry and hard					7	2	4					
6.7.55	Very dry and hard					5	5	4					
22.7.55	Very dry and hard					2	3						
4.8.55	Very dry and hard					4	2	5					
17.8.55	Very dry and hard					3		6					
2.9.55	Very dry and hard					4	2	5	1				
14.9.55	Moist and soft					1	6	5	1				
28.9.55	Moist and soft						8						
14.10.55	Moist and soft						3	2			1		
26.10.55	Dry and soft						14						
10.11.55	Moist and soft					1	26	2	2			12	
23.11.55	Soft						15					1	
7.12.55	Dry and soft					1	9	2	2			10	
21.12.55	Soft					8	20	1	1			10	
4.1.56	Soft and moist					1	8					11	
18.1.56	Soft and moist						9					6	
1.2.56	Dry and frozen					2	12		3			11	
15.2.56	Dry and frozen						4					1	
29.2.56	Dry and hard					1	6		2	4		11	
15.3.56	Soft and moist					1	10					4	1
29.3.56	Dry and hard						6			1		4	1
12.4.56	Dry and soft						25						
26.4.56	Soft and moist						14			1		5	3

100 pellets of sheep faeces were placed on a grass plot on 13.4.55. Each pellet contained 33 eggs of *N. filicollis* and 25 eggs of *N. battus*.

RESULTS

The protocols of this experiment occupy some twenty-seven tables similar to tables 3 and 4 and are not, therefore, given in full but are summarised by means of five tables. Table 1 shows the rate of development of the larvae within the eggs and time of survival of the free larvae of *N. filicollis*. Table 2 gives similar data for *N. battus*. In Table 3 are given full details of the examination of samples from plot 1 to illustrate the course of development of eggs in faeces voided on to the pasture in the spring. Table 4 gives similar data for plot 3 illustrating the development of eggs voided in the autumn. Finally, Table 5 summarises the meteorological observations made during the experiment.

TABLE 4

RATE OF DEVELOPMENT OF EGGS OF *NEMATODIRUS* SPP. WHICH WERE PLACED ON GRASS DURING SEPTEMBER

Date of collection of faeces	*State of faeces*	N. filicollis						N. battus					
		No development	*Early segmentation*	*Morula stage*	*Tadpole stage*	*Third stage larvae*	*Free larvae*	*No development*	*Early segmentation*	*Morula stage*	*Tadpole stage*	*Third stage larvae*	*Free larvae*
14.10.55	Soft and dry		(96)					21					
26.10.55	Soft and moist		(91)					38					
10.11.55	Soft and moist		62	24				29					
23.11.55	Soft and moist		34	47				28					
7.12.55	Moist	3	(103)					45					
21.12.55	Soft		(77)						(26)				
4.1.56	Hard and moist		(150)						(53)				
18.1.56	Soft and moist		(148)						(53)				
1.2.56	Dry and frozen		(89)						(23)				
15.2.56	Dry and frozen		(82)						(29)				
29.2.56	Soft and moist		(46)						(11)				
15.3.56	Soft and moist		(53)						(15)				
29.3.56	Soft and moist		(47)						(7)				
12.4.56	Dry and soft		1	68					(11)				
26.4.56	Soft and moist			12	1	14			(4)				
10.5.56	Soft and dry		1	2		64			(7)				
23.5.56	Soft and dry			3		94			(28)				
7.6.56	Soft and moist			1		4	1		(5)				
21.6.56	Soft and dry					41	4		1	7		2	
4.7.56	Soft and moist					34	3		1			7	
18.7.56	Soft and dry						100						
1.8.56	Crumbly and moist					11	6		2			1	
15.8.56	Soft and dry					15	3					1	2
29.8.56	Soft and moist					39	6					2	
12.9.56	Crumbly and moist					20	11					5	1
26.9.56	Crumbly and dry					2	3						

Pultaceous mass of faeces made from 100 pellets was placed on a grass plot on 28.9.55. Each pellet contained 101 eggs of *N. filicollis* and 27 eggs of *N. battus*.

TABLE 5

SUMMARY OF METEOROLOGICAL DATA COLLECTED AT WEYBRIDGE DURING THE EXPERIMENT

Month	*Temperature* (°F)			*Precipitation*	
	Maximum	*Minimum*	*Mean*	*Total (inches)*	*Days*
April, 13–30th, 1955 ..	66	30	48	0	0
May, 1955	68	36	51	3·40	12
June, 1955	73	41	59	2·30	6
July, 1955	82	46	64	0·25	3
August, 1955	86	39	64	1·48	7
September, 1955 ..	78	37	57	1·68	12
October, 1955	67	25	46	3·03	10
November, 1955 ..	59	24	42	1·54	6
December, 1955 ..	53	26	42	1·32	13
January, 1956	Records not available			3·92	17
February, 1956 ..	50	26	31	0·15	13
March, 1956	57	28	43	0·33	10
April, 1956	55	35	44	1·09	11
May, 1956	70	40	55	0·27	5
June, 1956	74	46	60	1·81	14
July, 1956	83	47	62	3·81	12
August, 1956	72	42	58	3·67	17
September, 1956 ..	76	44	59	2·59	10
October, 1956	63	33	50	1·80	15
November, 1956 ..	56	19	42	0·83	6
December, 1956 ..	55	30	43	3·03	13
January, 1957	55	28	42	1·32	12
February, 1957 ..	55	29	45	2·90	17
March, 1957	68	28	49	0·78	11
April, 1957	64	29	48	0·16	3
May, 1957	70	33	52	1·25	11
June, 1957	89	42	61	0·78	10

Some of the more important findings of the experiment can be summarised as follows. Both faecal pellets and pultaceous faeces appear to be equally good media for the development of the eggs of *Nematodirus* spp. The eggs of *N. filicollis* develop at a much faster rate than those of *N. battus* and it is also interesting to note that other observations not recorded in this paper show *N. spathiger* to develop even more quickly than *N. filicollis*. Eggs of *N. filicollis* voided on to the pastures in spring or summer can be expected to contain fully developed third stage larvae in four weeks and to have hatched in eight weeks. Development is arrested during the late autumn and winter and eggs passed during these seasons will remain in the early segmentation or morula stage until the spring when development recommences. With *N. battus*, however, eggs voided on to the pasture in spring will not contain fully developed third stage larvae until four to six months later. Hatching rarely takes place during the same year and usually does not occur until the following spring. Eggs of *N. battus* passed out by an animal during the autumn or winter remain in the early segmentation stage or morula

stage over winter. In the spring further development takes place and third stage larvae will be present in the eggs during the course of the summer. Hatching is, however, frequently delayed until the spring following the recommencement of development.

DISCUSSION

The ability of the preparasitic stages of *Nematodirus* spp. to survive the winter in various parts of the world has been observed by a number of workers including Griffiths (1937) in Canada, Kates (1943) in Maryland, Hawkins, Cole and Kline (1944) in Michigan, Todd, Kelly and Hansen (1949) in Kentucky, and Kates (1950) and Turner (1953) in Maryland. Most of these workers arrived at their conclusions from grazing experiments on pastures previously contaminated either naturally or artificially with the eggs of *Nematodirus* spp. and in most instances were not able to say precisely which of the several preparasitic stages were able to survive the winter, although Turner (1953) was able to demonstrate that the infective larvae of *N. spathiger* were able to survive the winter on pasture in Maryland. In the present experiment it was observed that all stages from the egg containing a segmented ovum to the free infective larva could survive winter conditions in the South of England.

The very slow development of *N. battus* followed by mass hatching of eggs in the spring has a bearing on the epidemiology of nematodiriasis. This phenomenon has been previously recorded by Zawadowsky and Zvjaguintzer (1933) who demonstrated that the eggs of *N. helvetianus* could survive the winter in Moscow and could complete their development and hatch in the warmer months of spring. These workers concluded that the llamas they were studying were subjected to an intensive invasion by great numbers of larvae which hatched in the spring with the result that the number of eggs of *Nematodirus* in their faeces increased steeply one month later. Outbreaks of nematodiriasis seen in young lambs in this country doubtless arise in a similar way. Thomas and Stevens (1956) reported a sudden increase in the spring in the numbers of infective larvae of *Nematodirus* spp. (chiefly *N. battus*) on pastures which had been contaminated with eggs of *Nematodirus* spp. the previous year. Unpublished work still in progress at this laboratory has confirmed this result. From the work reported in the present paper there is little doubt that the spectacular rise in pasture larval count which takes place in the spring derives from eggs which have overwintered in a partially developed state and which are stimulated to continue their development and to hatch in the spring. Although there are indications that temperature is involved, the exact stimulus which causes the dormant eggs to hatch in the spring has not yet been discovered but work is in progress which it is hoped will reveal it.

The advice, based on epidemiological observations, that outbreaks of nematodiriasis may be avoided by ensuring that lambs graze only on pasture which did not carry lambs the previous year

is shown to be soundly based. From the present work it is seen that contamination of pastures in autumn and winter does not contribute to the hazard the following spring and for practical purposes control can be achieved by avoiding those pastures likely to have been contaminated in the spring of the previous year.

CONCLUSIONS

Faecal pellets containing eggs of *N. filicollis* and *N. battus* were placed on grass plots one foot square at monthly intervals over a period of fourteen months, and the survival of the eggs and larvae on each plot was observed for the following year. The eggs of *N. filicollis* were observed to develop more rapidly than those of *N. battus;* the eggs of the latter do not hatch, at the earliest, until the spring following their arrival on the pasture. All the various preparasitic stages appeared to be capable of surviving the winter. The significance of these findings in the control of nematodiriasis is discussed.

ACKNOWLEDGMENT

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STUDIES ON THE HETEROPHILE ANTIBODIES ASSOCIATED WITH HELMINTH INFECTIONS

II. Heterophile Antibody in *Ascaris lumbricoides* Infection in Pigs

By

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INTRODUCTION

A heterophile antibody resembling the Forssman antibody has been noted in pig sera by Coombs (1954) and has also been referred to by Buchbinder (1935). Hitherto such heterophile anti-sheep erythrocyte antibodies occurring in the serum of normal animals have been considered as "normal" antibodies, as for example, the "natural" anti-sheep cell antibody in cattle (Rice and Duhamel, 1957) and that of rabbits (Mackie, 1925).

In a previous publication (Soulsby, 1958) it was shown that Forssman antibody is produced in rabbits infected with *Ascaris lumbricoides*, and furthermore that Forssman antibody raised by more conventional antigens, e.g. guinea pig kidney and fowl red blood cells, could be absorbed by certain adult or larval materials from *A. lumbricoides*. It appeared pertinent to enquire whether infection with *A. lumbricoides* could alter such "naturally" occurring heterophile antibody levels in pigs. In this communication experiments are reported in which it is established that, at times, high levels of heterophile antibody are associated with *A. lumbricoides* infection in pigs.

MATERIALS AND METHODS

Animals. Two groups of pigs were used. The first group of four was obtained from a reputed *A. lumbricoides*-free herd and was kept after weaning in thoroughly cleansed pens. The second group of five, obtained by Caesarian section, was raised in sterilised cages on an artificial diet and deprived of colostrum; it was designated the "disease free" group. Infections with *A. lumbricoides* were commenced at 12 weeks of age in the first group and 17 weeks of age in the second group.

Blood samples were taken from ear veins and serum was collected after overnight separation. Serum was stored at less than —20°C and before use in serological tests was inactivated at 56°C for 30 minutes.

Parasite materials. A. lumbricoides eggs for infection and larvae for absorption tests were prepared as previously described (Soulsby 1958).

Serological tests. In order to detect heterophile sheep cell antibody two main tests were used, a haemolytic test and a conglutination test. An anti-globulin test was found satisfactory but was too laborious for use with large numbers of serum samples.

The haemolytic test was based on the 50 per cent end point technique

with colorimetric estimation of haemolysis of a standard suspension of sheep red cells equivalent to 0·5 ml. of a 2·5 per cent suspension in the presence of four 50 per cent units of guinea pig complement. A detailed account of this method is given by Taliaferro and Taliaferro (1950).

The conglutination technique was adopted since its basic principle is to enhance the activity of a specific antibody, undetectable by ordinary tests, by the addition of horse complement and conglutinin. Such a technique has been designated the "direct conglutination reaction" by Hole and Coombs (1947a). Since pig serum is frequently markedly anti-complementary to horse complement (Coombs, 1954) it was necessary to modify the conglutination technique by sensitizing sheep cells to pig serum in the absence of complement and conglutinin. After sensitization the cells were washed once in a large volume of saline and after centrifuging and removal of the supernatant saline, were then resuspended in their original volume. Thereafter, complement and conglutinin were added and the test was completed in the usual way. Parallel trials with direct tests and "interrupted" tests showed that with high titre sera no advantage was gained by using the "interrupted" test. Since, however, the titres to be expected were unknown, it was considered that the use of the more laborious "interrupted" test was justified in order to avoid fallacious results should low titre sera be encountered.

Tests were performed in 50 mm. × 5 mm. tubes, unit volumes of reagents being 0·2 ml. All dilutions of sera and other reagents were made in veronal buffer (Mayer, Osler, Bier and Heidelberger, 1946). Sheep erythrocytes were washed three times in an excess of isotonic saline and used in a 0·4 per cent suspension in veronal buffer. Horse complement and bovine conglutinin, absorbed with sheep red cells to remove sheep cell antibody, were standardized to contain two minimal conglutinating doses following the methods described by Hole and Coombs (1947b). Tubes were incubated at 37°C for 30 minutes and were then centrifuged for about 2 minutes at 1,000 r.p.m. The degree of conglutination, determined by the facility of resuspension of the centrifuged cells, was arbitrarily designated ++++, +++, ++, + and negative, the ++ reaction being taken as an end point.

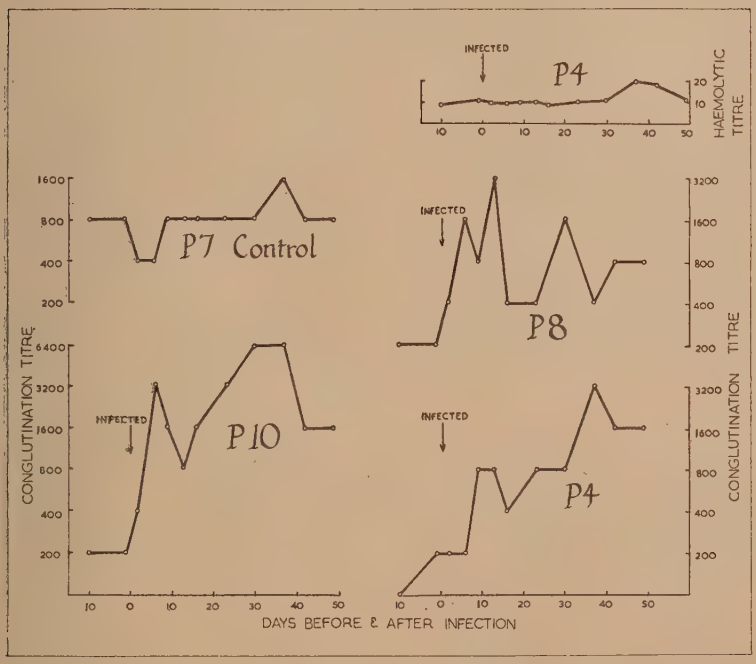
RESULTS

In the first group of pigs (P4, P7, P8 and P10), P7 served as an uninfected control, P8 and P10 received a single dose each of 300,000 *A. lumbricoides* eggs and P4 received 1,000 eggs daily for 5 days. The resulting heterophile antibody levels, as determined by the conglutination test, are illustrated in Fig. 1, which also shows the haemolytic heterophile antibody level in pig P4. All three infected pigs showed a rise in heterophile antibody within 10 to 15 days after infection with *Ascaris*. It is noteworthy that the haemolytic test was unsatisfactory for detecting this antibody; this point will be dealt with later.

The second group of pigs (P16, P17, P18, P19 and P20), the so-called "disease free" group, were infected with *Ascaris* when 17 weeks of age. P18 and P19 each received 1,000 eggs daily for five days, P20 received a single dose of 500,000 eggs whilst P16 and P17 served as uninfected controls. The resulting heterophile antibody

levels, determined by the conglutination technique, are illustrated in Fig. 2. As in the first group of pigs, heterophile antibody levels rose following infection, in one case (P18) to exceedingly high levels, reaching peak titre about the 15th day after infection. It is of note that in these pigs, especially the controls, the preinfection titres were particularly high. A similar situation in regard to the heterophile antibody in rabbit serum has been noted previously (Mackie, 1925; Taliaferro and Taliaferro, 1950; Soulsby, 1958).

Fig. 1



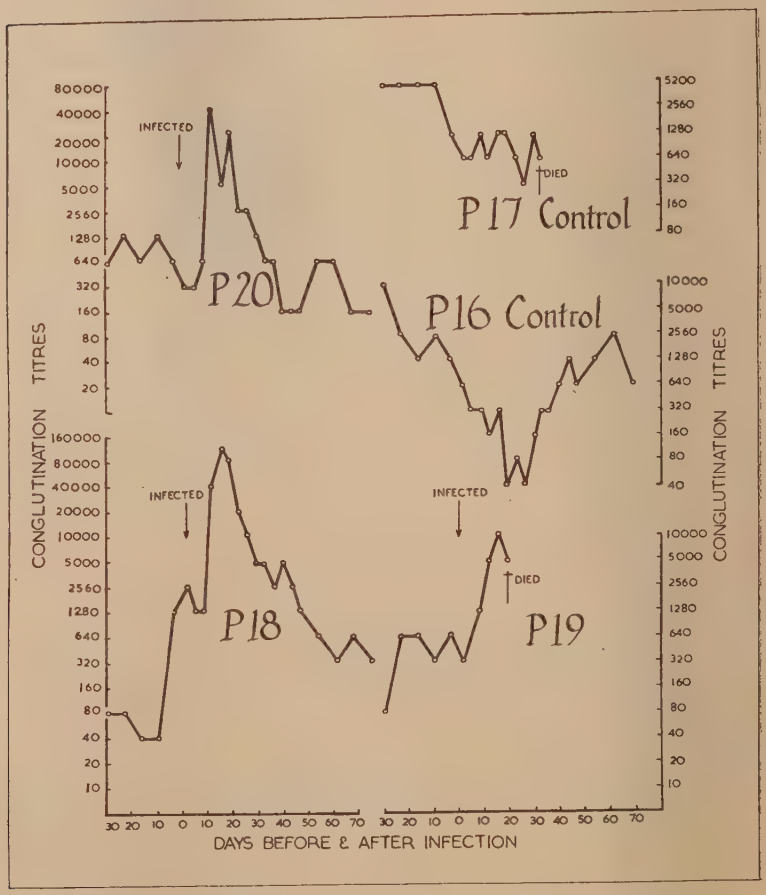
Titres of heterophile antibody as measured by the conglutination test in four pigs. P7 not infected; P8 and P10 infected with a single dose of 300,000 *A. lumbricoides* eggs; P4 infected with 1,000 eggs daily for 5 days. Figure includes the haemolytic heterophile titre for pig P4.

Identity of the Antibody

Since an antibody was being detected by the use of sheep erythrocytes it seemed likely that it would be related to the Forssman antibody. Absorption with ground up guinea pig kidney and fowl erythrocytes removed the antibody and no agglutination of sheep cells occurred using simple mixtures of serum and cells. Little other heterophile activity was detected since fowl erythrocytes, for example, were not agglutinated, whilst pigeon erythrocytes were agglutinated at low titres only (maximum titre recorded was $\frac{1}{10}$).

A heterophile antibody causing agglutination of pigeon erythrocytes is not regarded as true Forssman, but rather as a bird erythrocyte heterophile antigen associated with haemorrhagic septicaemia organisms (Buchbinder, 1935). It is possible, therefore, that two heterophile antibodies existed in the sera of these pigs, and subsequent work on other pig sera has shown this to be true. The low haemolytic ability of the main antibody under consideration prevents its immediate identification as Forssman. However, Talmage and Freter (1956) have shown that immunization of rabbits with sheep erythrocytes induces two Forssman antibodies, one with the classical

Fig. 2



Titres of heterophile antibody as measured by the conglutination test in five "disease free" pigs. P16 and P17 not infected; P18 and P19 infected with 1,000 *A. lumbricoides* eggs daily for 5 days; P20 infected with a single dose of 500,000 eggs.

Forssman haemolytic features, and another with a very low haemolytic ability but with good combining properties. The two can be separated by techniques using starch electrophoresis (Stelos and Talmage, 1957) and also by ultracentrifugal analysis (Talmage, Freter and Taliaferro, 1956) in which the haemolytic portion is of high molecular weight and the low haemolytic but combining portion is of low molecular weight. It is suggested that the antibody in question here resembles the low molecular type Forssman and that under conditions of the stimulation of heterophile antibody in the pig this type of antibody largely predominates. It should be noted, however, that the pig is apparently able to form haemolytic antibody since Hoerlein (1957) has induced such in pigs by the injection of sheep red cells.

The apparently low molecular weight Forssman antibody in pigs could be absorbed almost completely by either an extract of disintegrated third stage *A. lumbricoides* larvae or the residue from such an extract. A similar extract of *Oesophagostomum dentatum* third stage larvae produced similar results; subsequent experiments with *Oesophagostomum* infection in pigs, to be reported later, have shown that this parasite also may be a cause of elevated heterophile antibody levels in pigs. It was shown previously (Soulsby, 1958) that preparations of *Ascaris* larvae would absorb haemolytic Forssman antibody of rabbit origin. Since the non-haemolytic type in pigs is also absorbed by such materials it is probably the same antigen which is responsible in both antibodies.

Relationships to Other Antibodies

In rabbits it was found that Forssman antibody levels and true anti-*Ascaris* antibody levels closely paralleled each other (Soulsby, 1958) and thus the Forssman antibody could be regarded as a natural "marker" antibody. Tests carried out on the sera used in this investigation have shown the same to be true with regard to pigs.

DISCUSSION

The demonstration that heterophile antibody levels increase following infection of pigs with *A. lumbricoides* extends earlier observations in rabbits to the natural host of the parasite. Consequently the presence of such a heterophile antibody in the sera of pigs in a normal environment can, in part, be legitimately attributed to natural infection with this parasite. Nevertheless, other stimulants to heterophile antibodies must occur since in artificially reared and worm-free pigs substantial titres were found in controls and also prior to infection in infected pigs. These may be due, in part, to *O. dentatum* infection, and almost certainly to bacterial infection, since Buchbinder (1935) gives an extensive list of organisms which carry the Forssman antigen.

In rabbits the haemolytic Forssman antibody has been used as a

natural "marker" antibody (Soulsby, 1958) and it may be possible to put the heterophile antibody in pigs to similar use. Using such a concept it is seen that in the first group of pigs a secondary rise in heterophile antibody occurred roughly a month after infection. Initially it was thought that this might be due to late larval stages establishing themselves in the bowel. Unfortunately, the second group of pigs did not show this secondary rise, but further work is in progress to ascertain whether larval stages in the bowel can cause stimulation of antibody. As an alternative to this the secondary rise might be attributed to the disintegration of larvae trapped in the tissues during migration. A similar secondary rise in rabbits infected with *Ascaris* was ascribed to this (Soulsby, 1958). It should be further considered that this "secondary" rise may be due fortuitously to bacterial infection.

The work has shown that the conglutination test is extremely satisfactory for the detection of antibody, often present in high titres, and which otherwise would have been undetected by more straightforward techniques. It may be that many antibodies exist in the sera of domestic animals which possess good combining powers but which are undetectable by conventional methods, and that suitable modification of the direct conglutination test and its reagents would make them demonstrable. It is certain that haemolytic tests using guinea pig complement are unsatisfactory for the detection of this antibody. Haemolytic titres can be increased slightly by performing "interrupted" tests to avoid the anti-complementary action of pig serum. Coombs (1954) mentions that the anti-sheep red cell antibody of pig serum fails to absorb guinea pig complement when combined with sheep erythrocytes, and it may be that other complements would be more successful in establishing a haemolytic test. It seems more likely, however, that this antibody is comparable to that described by Talmage *et al.* (1956) being of low molecular weight and low haemolytic ability, yet possessing the other properties of true Forssman antibody.

The significance of such a heterophile antibody in pigs infected with *A. lumbricoides* is difficult to ascertain. Buchbinder (1935) has suggested that heterophile antibodies may possess some protective power in respect to infection. In the present work, however, relatively high titres of heterophile antibody did not prevent or mitigate infection in pigs and it is most probable that the heterophile antigen is an unimportant one in *A. lumbricoides*. It is presumably unconnected with the essential metabolic processes of the parasite which are almost certainly incapacitated when immune mechanisms retard or halt parasite development within the host. Under artificial conditions the injection of sheep erythrocytes into pigs may induce conditions of anaphylactic shock due to the presence of heterophile antibody, a consequence which has been mentioned by Hoerlein (1957).

CONCLUSIONS

Infection of pigs with *A. lumbricoides* stimulates the production of a heterophile antibody, detectable by the direct conglutination test, and sometimes reaching high levels. This antibody, which follows the course of other anti-*Ascaris* antibodies, has a very low haemolytic ability and is thought to be comparable to a low molecular weight antibody seen in rabbits following injection of sheep cells.

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PARASITIC BRONCHITIS IN CATS DUE TO THE NEMATODE *ANAFILAROIDES ROSTRATUS* GERICHTER, 1949*

By

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INTRODUCTION

In a survey of the helminth infections in cats in the Kandy district, Ceylon, it was found that over 60 per cent were infected with the nematode *Anafilaroides rostratus* Gerichter, 1949 (Seneviratna, 1955). Apart from Ceylon this nematode has been recorded only from Palestine, where one of seventy-three cats examined was found to be infected (Gerichter, 1949). The lesions caused by this parasite have not been described.

Most of the cats examined in Ceylon were stray animals that had predatory instincts and none of the animals below six months of age was found to be infected. Biological studies of the first stage larvae indicate that they cannot withstand freezing, high temperatures or desiccation (Seneviratna, 1957) so that the distribution of the worm is likely to be limited to semi-tropical or temperate zones where sub-zero temperatures do not occur and where there are no long dry periods or high temperatures. Such conditions are present in the Kandy district of Ceylon, where the rainfall is about 80 to 120 inches per year and the temperature varies from 75° to 93°F. The limited geographical distribution is explained by the very delicate nature of the first stage larva. The fact that it can use unrelated molluscs, such as *Achatina fulica, Mariella dussumieri* and the British snail *Helix aspersa* as intermediate hosts (Seneviratna, 1957) would indicate that it could probably establish itself if introduced into any new region provided suitable climatic conditions obtained. It appears to be more adaptable to different intermediate hosts than to different climatic conditions.

Once *A. rostratus* becomes established in an area it can become abundant if climatic conditions are suitable because it can use many transport hosts, such as mice and chickens (Seneviratna, 1957). When a cat becomes infected it harbours the parasite for over 313 days and during most of the period, larvae are being constantly excreted. Infestation of young cats is not seen because the prepatent period is long (78 days) and clinical evidence of infection is not quickly detected since cats usually become infected by eating the intermediate or transport hosts, which they rarely do until after weaning. Clinical evidence of infection is thus seen at the earliest

* Part of a Thesis approved by the University of London for the award of the Ph.D. Degree.

in cats four to five months old, and the older the cat the greater are its chances of acquiring and retaining the infection.

A. rostratus lives in the peribronchial tissue and the bronchial wall (Seneviratna, 1957). The common lungworm *Aelurostrongylus abstrusus* (Railliet, 1898) lives in the lung parenchyma or the respiratory bronchioles and causes a chronic verminous pneumonia with nodule formation. The condition has been described and reviewed by Blaisdell (1952). Further information has since been published by several authors (Gustafson, 1954; William, 1955; Mackerras, 1957). Because *A. rostratus* lives in the peribronchial tissue and the bronchial wall, the lesions it produces are different from those caused by other worms that live in the bronchial lumen or in the lung parenchyma of domestic animals. The lesions are, however, analogous with those seen in parasitic tracheobronchitis in dogs caused by *Oslerus osleri* (Cobbold, 1879) Hall, 1921 and described by Blumberg (1882), Hare (1930) and Urquhart, Jarrett and O'Sullivan (1954). Tracheobronchitis in dogs is a proliferating type of lesion, resulting in the production of nodules located in the mucosa of the trachea at its bifurcation and of the larger bronchi. The adults are seen only in the nodules, though the larval forms and the immature ones may be seen in the lymphatics and the pulmonary alveoli (Urquhart *et al.* 1954).

A bronchitis similar to the one seen in *A. rostratus* infection may, however, be caused by the nematode *Vogeloides massinoi*. This parasite was seen in the walls of the bronchi of 30 per cent of cats in Armenia (Davtian, 1933), but there is no published account of the lesions which it causes. A *Vogeloides* species has been found in the lungs of cats from Madras, India (Alwar, 1957) but the lesions produced were not described. The type of bronchitis produced by *A. rostratus* in cats is described below.

MATERIALS AND METHODS

The pathological changes were studied in cats infected naturally and experimentally. The lesions and blood pictures in the infected animals were compared with those of control cats which were negative to infection and were killed by the same method. It was observed that euthanasia by injection of Nembutal was more satisfactory than inhalation of chloroform since the latter caused some degree of oedema in the lungs.

Sections were prepared in the usual manner and stained with haematoxylin and eosin, Weigert's stain for elastic fibres, or van Gieson's stain. Haematoxylin and eosin were the most satisfactory stains and were employed in most of the cases.

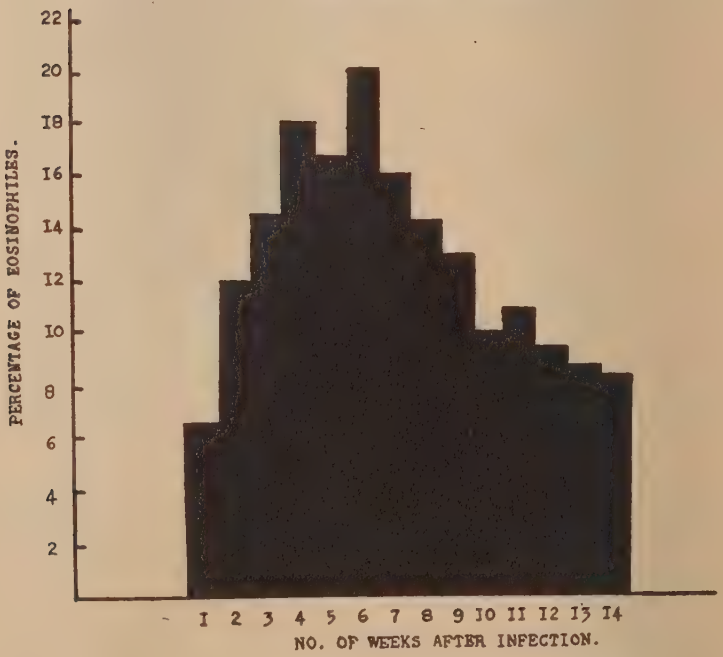
The pathological changes were studied in six cats which were infected experimentally by feeding each about 50 to 130 infective larvae of *A. rostratus* obtained from artificially infected molluscs. These changes are summarised in Table 1. The animals were killed at various intervals from the 19th to the 313th day of infection. It was thus possible to trace the pathological changes as the helminth grew from the larval stages to the adult. The pathological changes in natural infections were studied in three cats.

RESULTS

Stages of Infection

In the early stages of the infection, when the larval forms were developing in the lungs, there was leucopenia of a few weeks duration followed by leucocytosis which was accompanied by eosinophilia which was marked if the infection was heavy (Histogram 1). The eosinophil count remained high so long as infection persisted. The histopathological changes in the lung due to the presence of the parasite may be divided into four stages: the invasive stage, the progressive stage, the infective stage, and the regressive stage.

HISTOGRAM I, SHOWING THE EOSINOPHILIA IN CAT NO.42.
(EXPERIMENTALLY INFECTED).



Invasive stage. As the larvae grow there is very slight initial leucocyte response and a slight cellular reaction round them. Only a few neutrophils are seen, the other cells being eosinophils and mononuclear cells. These changes were seen in cats 11 and 43.

Progressive stage. During this stage there is mild bronchitis and peribronchitis (Figs. 1 and 2) and in areas where there is heavy infection, the columnar epithelium is partly destroyed and the basement membrane becomes disorganised with displacement of the bronchial glands. These changes were seen in cats 15, 16 and 43.

Infective stage. At this time the worms have attained full sexual maturity and start producing larvae. There is an increase of fibrocytes and fibrous tissue around the bronchi. The bronchial mucosa and the glands around the helminths undergo hyperplasia and hypertrophy (Fig. 3) and the changes in the previous stage are partly repaired. The worms become enclosed in a thin fibrous tissue capsule and the mild leucocyte response remains. These changes were seen in cats 38 and 42. In very heavy infections, the lumen of the bronchi may be partly obliterated by plugs of mucus and larvae, as in cat 38. In this case there was pneumonia in the right cardiac lobe, with areas of atelectasis and compensatory emphysema. Such cases are rare and are seen only in heavy infections. The principal lesion is a bronchitis and peribronchitis.

Regressive stage. These changes were seen in one of the naturally infected cats (38) and in cats studied in the survey (Seneviratna, 1955). The worms live for over a year after which some of them may die. When a worm in a fibrous tissue capsule dies there is a great accumulation of leucocytes, especially polymorphs (Fig. 4), but the leucocytes are unable to penetrate the fibrous tissue capsule. In one section, a calcified worm was seen. Some of the other changes seen in the third stage, such as fibrosis, progress further. In natural infections of the old animals, though a few worms were seen to have died, the majority were alive.

Symptomatology

This was studied in the experimentally infected cats 11, 13, 15, 16, 42 and 43 as well as in naturally infected cats 36, 37 and 38. These cases are summarised in Table 1. In some of the former there was a slight cough about the second or third week after infection which either persisted or gradually subsided. None of the experimentally or naturally infected cats suffered badly except cat 38 which showed signs of pneumonia. In some of the experimentally infected cats there was a slight rise in temperature from the third to fifth week but it soon subsided. One animal showed lethargy and one heavily infected cat (36) showed greater opacity of the bronchial region on radiological examination. This cat also showed a mild occasional cough.

Therapeutic Trials

These were conducted in naturally infected, stray cats brought to the laboratory and kept there. In these trials three drugs, known to have anthelmintic properties or used in the treatment of lungworm infections, were tried, viz.: piperazine hydrate ('Antepar Elixir' Burroughs Wellcome & Co.), sodium iodide in aqueous solution given parenterally, and Antimosan Bayer (Stibophen B.P.). Piperazine and its related compounds have been widely used in the treatment of filariasis and other helminth infections in man and animals. Sodium iodide has also been used in the treatment of lungworm infections

in domestic animals (Cameron, 1933). Antimosan was first used in the treatment of human schistosomiasis and has since been used in a variety of helminth infections, notably filariasis (Faust, 1949). Malherbe (1954) found it effective against *Oslerus osleri*, a nematode closely related to *A. rostratus*. (Seneviratna, 1957).

TABLE 1

SUMMARY OF PATHOLOGICAL CHANGES AND SYMPTOMS SHOWN BY CATS EXPERIMENTALLY OR NATURALLY INFECTED

No.	*Approximate no. of infective larvae fed*	*Days before euthanasia*	*Symptoms*		*Pathological changes and stage of larva present*
			Cough	*Elevation of temperature*	
11	50	19	—	—	Probably 4th stage in lung. Mild leucocyte response round them.
13	50	46	—	—	Immature helminths. Leucocyte response and mild localised bronchitis.
15	100	56	Slight, from 3rd week.	By 1–2°F from 3rd–5th week.	Immature helminths. Eosinophilia, leucocyte response and localised bronchitis.
16	100	58	Mild, from 4th week.	—	Non gravid females. Same changes as in No. 15.
36	Natural heavy infection.	—	Occasional	—	Eosinophilia; radiograph revealed opacity of bronchial region. Euthanasia not done.
37	Natural infection.	—	—	—	Eosinophilia. Euthanasia not done.
38	Natural infection.	—	Cough and difficult breathing.	Slight.	Pneumonia with atelectasis and emphysema. Bronchitis and peribronchitis with fibrosis.
42	130	313	Slight.	Slight in 3rd week. Animal dull and lethargic.	Eosinophilia in 2nd week, gradually subsided. Initial leucocytosis. Bronchitis and peribronchitis. Leucocytic response mild.
43	50	74	Slight in 2nd week.	—	Eosinophilia. Histological changes as in cat No. 15.

Piperazine hydrate was given at the rate of 100 mg. per kg. body weight daily to three cats for 8 to 10 days. In two of the three cats there was a reduction in the number of larvae excreted in the faeces, while in the third and the control cats there was no reduction. All cats showed live adults after euthanasia. Sodium iodide was given as a 10 per cent aqueous solution either intravenously or intratracheally to three cats on alternate days for three to four days. The dosage employed was 2·5 ml. There was no appreciable reduction

in the number of the larvae excreted nor any action on the adults. Antimosan was given intramuscularly or subcutaneously to four cats at the rate of 0·2 ml. per kg. body weight every third day until four or five injections had been administered. A gradual reduction occurred in the number of larvae and on euthanasia some dead and disintegrating worms were seen in two animals. One animal, which was rather weak, died during the course of the treatment, probably due to the effect of the drug. Two animals showed depression and anorexia during the course of treatment. The fourth animal escaped during treatment.

DISCUSSION

Sodium iodide was not found to be effective either intratracheally or intravenously. The beneficial effects of this drug claimed by many workers in the treatment of lungworm infections are probably due to the effects of iodides in lowering the viscosity of the bronchial secretions and reducing the development of atelectasis (Baker, Roettig and Curtis, 1951).

Piperazine hydrate reduced the production of the larvae, but had no effect on the adults in the dosages employed. It is, however, possible that if the drug had been given for a longer period, as is done in the treatment of filariasis, or at a higher dosage rate there would have been an effect on the adults.

Treatment with Antimosan gave the most promising results, but the drug was rather toxic. If it had been given at longer intervals as is done in the treatment of filariasis the toxicity might have been less. As an alternative it would seem preferable to start with a lower dosage and gradually increase it. The results obtained in the present series are encouraging and merit further study.

CONCLUSIONS

Infection of cats with the nematode *Anafilaroides rostratus* causes a chronic bronchitis and peribronchitis with marked hyperplasia of the bronchial mucosa. Infected cats show only a mild cough. The infection has been recorded only in Palestine and in the Kandy district of Ceylon. Treatment with Antimosan given intramuscularly or subcutaneously at the rate of 0·2 ml. per kg. body weight produced encouraging results but the drug appeared to be rather toxic.

ACKNOWLEDGMENTS

It is a pleasure to thank my supervisor, Dr. J. N. Oldham, for his valuable suggestions and helpful criticism, Professor C. A. McGaughey of the Ceylon University and the authorities of the Royal Veterinary College for the facilities provided in Ceylon and London respectively, Mr. F. A. Shephard for the photomicrographs, and Mr. N. Weerasinghe and Mrs. B. L. Mackenzie for their technical assistance and the care of the experimental animals.

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LEGENDS TO ILLUSTRATIONS

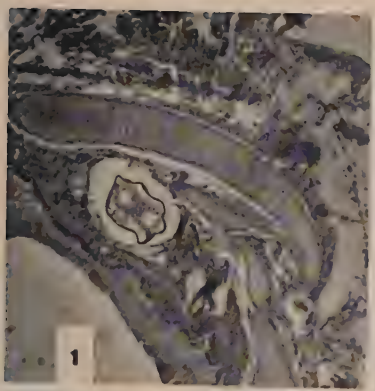
Fig. 1. Section of the lung of cat 15 showing an immature helminth. There is mild cellular reaction; the bronchial glands are displaced but the bronchial mucosa is intact. Stain H. & E. × 56.

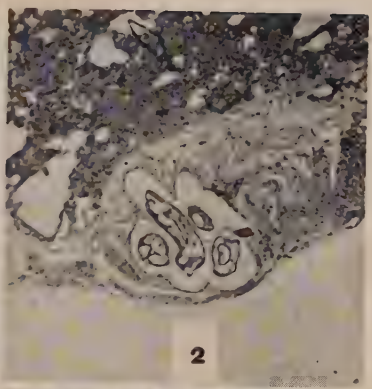
Fig. 2. Section of lung of cat 16 showing mature non-gravid female. There is disorganisation of the mucosa around the helminth. The bronchial glands have been displaced and the columnar epithelium eroded causing mild bronchitis and peribronchitis. Stain H. & E. × 32.

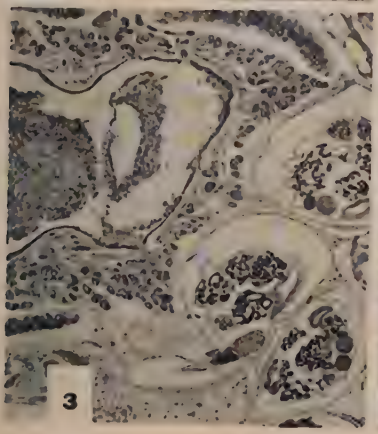
Fig. 3. Section of lung of cat 42 with adult worms *in situ.* They lie round the bronchial wall. There is chronic bronchitis and peribronchitis. The bronchial mucosa has undergone hyperplasia and hypertrophy. Stain H. & E. × 32.

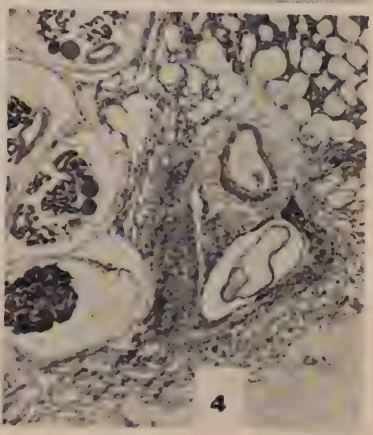
Fig. 4. Section of lung of cat 38 showing a degenerated worm which is surrounded by leucocytes among which polymorphs predominate. None of the leucocytes has been successful in penetrating the fibrous tissue capsule that surrounds the worm. To the left are seen sections of live worms, the leucocyte response round which is negligible. Stain H. & E. × 32.

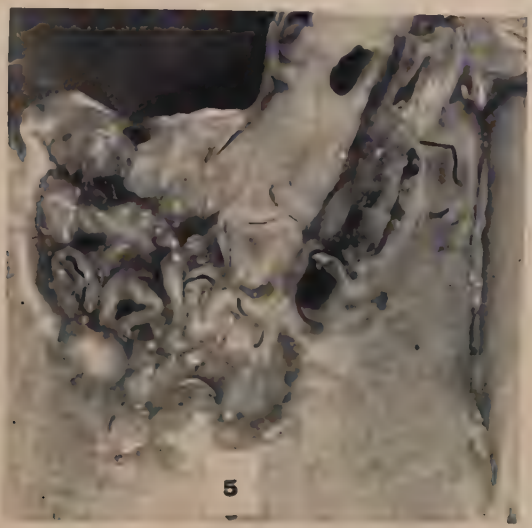
Fig. 5. Lung of cat 42 infected with *Anafilaroides rostratus.* The worm is intimately coiled round the bronchi, the intestine of the parasite showing as dark lines.











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SITE OF DEVELOPMENT OF THE LUNGWORM *MUELLERIUS CAPILLARIS* IN EXPERIMENTALLY INFECTED LAMBS

By

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INTRODUCTION

Muellerius capillaris is the commonest of the lungworms found in sheep in this country.

In a survey made from May, 1953 to May, 1954 as many as 99 per cent of the sheep slaughtered at Watford abattoir were infected (Rose, 1955). The adult worm was first discovered by Koch in 1883 and the life cycle elucidated by Hobmaier and Hobmaier in 1929. Subsequently the free-living and intermediate-host phases of the life cycle were studied by a number of workers. The parasite in the final host has, however, been more or less ignored.

It is well established that sheep pick up the infective lungworm larvae by accidently ingesting the intermediate host, which may be one of a number of species of slugs and land snails. The infective larvae are liberated from the mollusc's foot, in which they are localised, during the process of digestion. It is assumed that they then reach the lungs by passing via the mesenteric lymph glands, lymphatics and blood stream. This assumption is reasonable as a similar route is taken by the larvae of other species of lungworms (Hobmaier and Hobmaier, 1929; Soliman, 1953). Precisely what happens when the larvae reach the lungs is not, however, at all clear. Cameron (1933) has suggested that the adult worms probably live for part of their lives in the arteries and arterioles. This idea is repeated by both Lapage (1956) and Mönnig (1957). The former writes " The adult worms may live for a time in the smaller branches of the pulmonary artery by which they reach the lungs, but eventually they settle in the substance of the lungs". There is, however, no record of *M. capillaris* ever having been found in the arterioles.

The effects of *M. capillaris* on the health of the sheep have never been clearly demonstrated, but in view of its widespread distribution it is desirable to know more about the host-parasite relationship,and in particular to ascertain to what extent the lungworm is pathogenic. Experiments are now in progress at Weybridge with this in mind. In the course of a preliminary experiment designed to study the effects of the lungworm on the health of the sheep, it was possible also to make observations on the course and site of development of the worm. The present paper is an account of these observations.

METHODS

The common grey field slug *Agriolimax* is one of the molluscs which can act as the intermediate host for *M. capillaris*. The two species *A. reticulatus* and *A. agrestis*, which cannot be distinguished morphologically, have been used in our experiments as they can easily be kept in the

laboratory. A detailed account of the methods used in maintaining and infecting these slugs has been published elsewhere (Rose, 1957). One modification was introduced in the present work. As soon as the larvae had reached the infective stage, the slugs were kept in a refrigerator at a temperature of 8° to 10°C. as this reduced mortality to a minimum.

The infective larvae, localised in the slug's foot, can be counted when the foot is cut off, gently compressed between two glass slides and examined microscopically. To infect a sheep, one or more feet containing the requisite number of infective larvae were suspended in tap water and pipetted into the back of the throat, followed by more water to ensure that the foot was swallowed. The larvae were not removed from the foot as separation by mechanical means proved difficult and although it could be effected by artificial peptic digestion it was considered undesirable to subject the larvae to such a preliminary enzyme action.

The observations recorded in the present paper concern a group of four lambs, approximately four months old. Each was given 60 to 70 infective larvae on five consecutive days each week for a period of 44 weeks, after which the lambs were killed. At post mortem, lesions from both lungs were removed, each was teased out on a glass slide while being viewed through a dissecting microscope, a drop of 1 per cent aqueous eosin was added, another slide was placed on top and the preparation gently compressed. The eosin stained the lung tissues and disintegrating worms, whereas intact worms did not take up the stain and stood out against the coloured background. The eosin was particularly useful when examining the smallest lesions for fourth-stage larvae which are extremely difficult to find in an unstained preparation. Small pieces of healthy tissue were similarly examined and the bronchial tree was opened up for the detection of both mature and immature worms.

RESULTS

The lesions resulting from infection with *M. capillaris* can be very variable in size, ranging from less than 1 mm. to several centimetres in diameter. In an earlier paper (Rose, 1955) they were classified into three size groups, 'small', 'medium' and 'large'. All of those in the present observations were of the 'small' type being less than 5 mm. in diameter. They did, however, exhibit marked differences in structure and for the sake of simplicity were divided into three categories depending upon the extent of calcification, namely 'heavily', 'lightly' and non-calcified.

The smallest of the non-calcified lesions, which were little more than pin-point in size, contained fourth-stage larvae approximately 720μ in length in which no sexual differentiation was apparent. Intact worms which ranged from immature forms only slightly larger than the fourth-stage larvae to fully grown adults up to 25 mm. long were present in the larger non-calcified lesions. Adult worms were found also in the lightly calcified lesions. Some of these worms were intact whereas others exhibited signs of cytoplasmic degeneration and broke down when touched with a needle. In the heavily calcified lesions, only disintegrating worms were found. Worms were not found in either the healthy lung tissues or the bronchial tree.

It is evident that development of *M. capillaris* from the fourth-stage larva to the adult worm can take place within the lung tissues. It seems most probable that the lungworm larvae, which are approximately 32μ in width, cause the lung capillaries, which are of much smaller diameter, to rupture when the larvae are carried into them by the circulating blood. They then pass to the adjacent lung parenchyma or subpleural connective tissue where they develop to maturity.

If adult worms do live in the arteries and arterioles and then eventually settle in the substance of the lungs, as has been suggested, it ought to be possible to find migrating worms in healthy lung tissue and in the arterioles. Such migrating worms were not encountered either in the present observations or in the 1953 to 1954 survey. Occasionally adult worms are recovered from the bronchioles, but in the course of examining 370 lungs only two specimens were recovered from lungs in which the 'large' type of lesion was present. These 'large' lesions, which are not uncommon in adult sheep, contain a mass of eggs and first-stage larvae together with mature worms. The presence of the first-stage larvae appears to result in the breakdown of the tissues; consequently the larvae are liberated into the alveoli and bronchioles. It is most probable that the adult worms in these lesions are similarly liberated. The occasional worm found in the bronchioles has presumably made its way there in this manner and it is not to be regarded as an actively migrating worm. It seems unnecessary to postulate that the adult worms may live in the arterioles with a subsequent migration into the tissues of the lungs, particularly in the absence of any supporting evidence, and when the various stages of development from fourth-stage larva to mature worm can be demonstrated in the lung tissues.

An extremely interesting feature of the development of *M. capillaris* is the way in which many of the worms remain isolated, each in its own lesion. The majority of the lesions examined contained but a single worm intimately entwined within the tissues. Female worms so isolated cannot produce fertile eggs and first-stage larvae. It is even possible for all the worms in the lungs to be isolated each in its own lesion and this was in fact seen in two experimentally infected lambs. First-stage larvae were never recovered from their faeces, although on post-mortem examination, several hundred lesions were present in the lungs. Not one of the many lesions examined contained more than a single worm, most of which were fully grown. When first-stage larvae have been recovered from the faeces of other lambs it has always been possible on post-mortem examination to find a few lesions containing both male and female worms as well as first-stage larvae. It would seem that for the fertilis sation of the female worms to take place, the male and female worm-must come together at the outset and mature in the same lesion.

A point of practical significance which emerges from the present observations is that faecal examinations have very little meaning.

The absence of first-stage larvae from the faeces, even over a prolonged period, does not necessarily mean that the animal is free from infection. Furthermore, when larvae are present the numbers being passed cannot be related in any way to the degree of infestation since only a small percentage of the female worms present may be producing fertile eggs.

CONCLUSIONS

Four lambs were each given 60 to 70 infective larvae of *M. capillaris* on five consecutive days every week for 44 weeks. At the end of this period the lambs were slaughtered and the lungs examined.

It was established that development from the fourth-stage larva to adult worm takes place within lesions in the lung tissue.

The observations did not support the idea that the adult lungworms live for some time in the arterioles.

As a result of the reaction of the hosts' tissues enclosing the parasite within a lesion, many lungworms become isolated from each other: consequently not all the females succeed in producing fertile eggs. It is in fact possible for no fertile eggs and first-stage larvae to be produced even though many worms are present in the lungs. The examination of faeces is, therefore, of very limited use in diagnosing infections with *M. capillaris*.

ACKNOWLEDGMENTS

I wish to thank Dr. S. B. Kendall for helpful advice on the presentation of this paper, and Mr. J. Bailey for technical assistance.

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VALUE OF INTERRUPTED TREATMENTS AND PREVENTIVE MEDICATION IN THE CONTROL OF INTESTINAL COCCIDIOSIS CAUSED BY *EIMERIA NECATRIX*

By

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INTRODUCTION

Outbreaks of caecal coccidiosis caused by *Eimeria tenella* can in most instances be controlled successfully in Britain by the use of the sulphonamides, sulphadimidine and sulphaquinoxaline. The best results have been obtained with an interrupted treatment whereby a solution of the soluble sodium salt of the sulphonamide is administered to the flock for a period of three days followed by an interval of two days on plain water and a further three days of medication. For the prevention of outbreaks of caecal coccidiosis, as distinct from the control of established disease, there is an increasing use of either Nitrofurazone or more recently Nicarbazin, these preparations being mixed in the dry mash and fed continuously during the rearing period.

The object of the present study was to assess the effectiveness under experimental conditions of comparable types of treatment for the control of intestinal coccidiosis caused by *E. necatrix*.

MATERIALS AND METHODS

Chicks (R.I.R., W.L./R.I.R. and L.S./R.I.R.) were obtained as day-olds and were kept in isolation until required for experiment. They were maintained in metal wire-floored hovers and received water and food *ad libitum*. The sodium salts of sulphadimidine or sulphaquinoxaline were administered in the drinking water. Nitrofurazone or Nicarbazin was mixed in the dry mash.

Birds were infected with a single inoculum of sporulated oocysts of *E. necatrix* into the crop, the strain of *E. necatrix* being one which had been isolated and maintained at Weybridge (Davies, 1956). In order to ensure a uniform rate of infection in the various groups, all birds in each of the experiments (Figs. 1 to 6) were infected on the same day and treatment was commenced at intervals relative to the time of infection. The commencement of the first period of treatment occurred on the fourth day before infection (Group K), on the third day before infection (Group J) and so on to Group B where treatment commenced on the fourth day after infection. Group A served as an untreated control. For demonstration, however, the results have been recorded in a form which suggests that infection was contracted in different groups of birds at twenty-four hourly intervals over a period of ten days, the day on which each group contracted infection being marked in Figs. 1 to 6 by an $\times$. This way of recording the results is considered to indicate more clearly the effect of interrupted

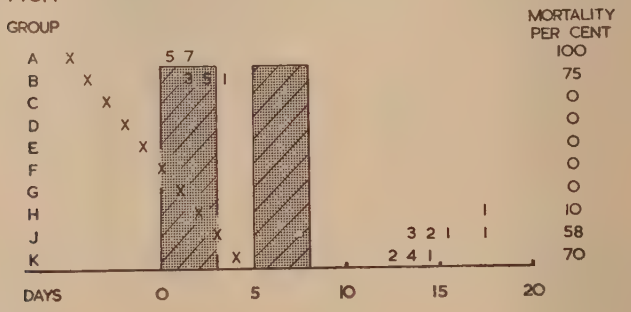
treatment on a field outbreak of intestinal coccidiosis which would be likely to be contracted by a flock over a period of days.

RESULTS

Effects of a 3 – 2 – 3 Interrupted Treatment

The results of an interrupted treatment with 0·2 per cent sodium sulphadimidine for 3 – 2 – 3 days i.e. 3 days on medicated water followed by an interval of 2 days on plain water and a further 3 days of medication, are shown in Fig. 1. Ten groups of two-weeks old chicks were infected with *E. necatrix*, each chick receiving 100,000 sporulated oocysts. Birds in group A acted as untreated controls and there was a 100 per cent mortality in this group. Birds in Group B were treated at the ninety-sixth hour after infection and the rate of mortality was reduced to seventy-five per cent. No deaths occurred in Groups C to G inclusive, where the two treatment periods successfully controlled the second generation schizogony of the parasite. In groups H, J and K, however, several deaths from acute intestinal coccidiosis were noted from five to ten days after the end of treatment.

FIG. 1

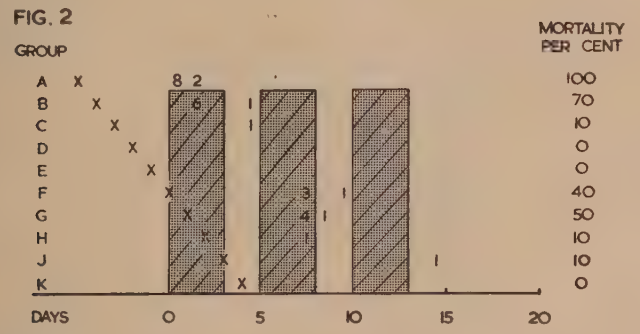


Interrupted treatment for 3–2–3 days, 0·2 per cent. Sodium Sulphadimidine. Twelve chicks per group. Groups G, H & K, ten only.
Shaded areas denote periods of treatment. Day of infection marked . .

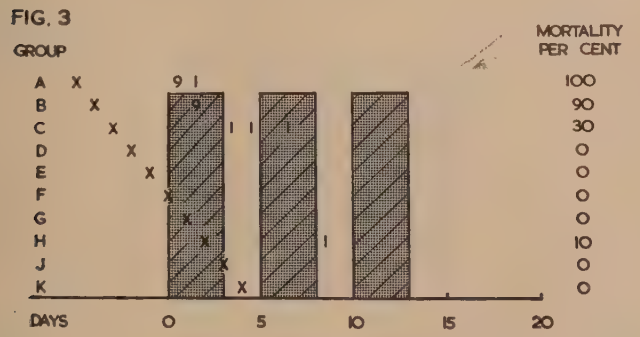
Interrupted Treatment for 3 – 2 – 3 – 2 – 3 Days

In an effort to prevent the re-appearance of intestinal coccidiosis in groups of treated birds a third course of treatment was given after a further interval of two days, i.e. treatment was now given intermittently for thirteen days. Figs. 2 and 3 show the results of such treatment with 0·2 per cent sodium sulphadimidine or 0·043 per cent sodium sulphaquinoxaline respectively. When two series of ten groups of two-weeks old chicks were infected with 200,000 sporulated oocysts of *E. necatrix* a number of deaths, which may be attributed to the more acute infection, were recorded in groups F and G (Fig. 2) which in the previous observation had suffered no mortality. In groups H. J and K (Figs. 2 and 3) however, the infection was

controlled by the additional treatment. There was only one death in group J (Fig. 2) on Day 15.



Interrupted treatment for 3–2–3–2–3 days, 0·2 per cent. Sodium Sulphadimidine. Ten chicks per group.



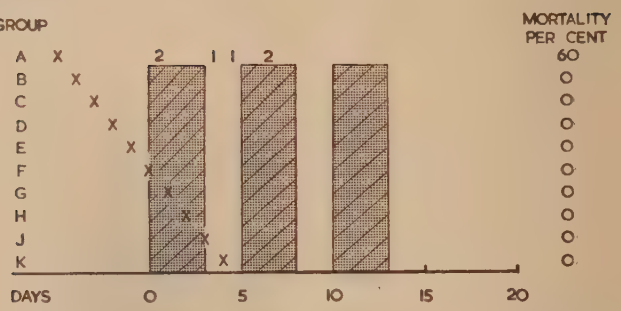
Interrupted treatment for 3–2–3–2–3 days, 0·043 per cent. Sodium Sulphaquinoxaline. Ten chicks per group.

Treatment at Reduced Concentrations

Under field conditions, the addition of a third period of treatment might increase the dangers of toxicity. In the following experiments the effects of an extended treatment with reduced concentrations of sodium sulphadimidine or sodium sulphaquinoxaline were observed.

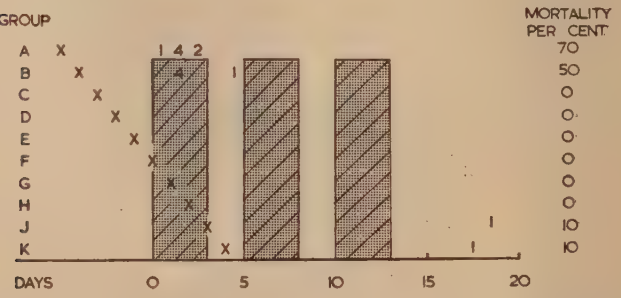
Three series of older birds were given thirteen days intermittent treatment as follows. Six-weeks old cockerels were infected with 50,000 sporulated oocysts and were treated with 0·15 per cent sodium sulphadimidine. Seven-weeks old mixed cockerels and pullets received 100,000 sporulated oocysts and were treated with 0·1 per cent sodium sulphadimidine. Six-weeks old pullets were infected with 100,000 sporulated oocysts and were treated with 0·032 per cent sodium sulphaquinoxaline. The results are shown in Figs. 4, 5 and 6.

FIG. 4



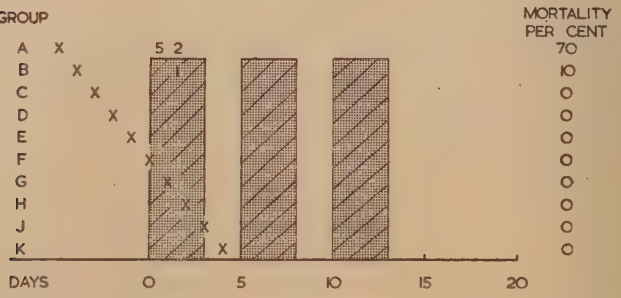
Interrupted treatment for 3–2–3–2–3 days, 0·15 per cent. Sodium Sulphadimidine. Ten cockerels per group.

FIG. 5



Interrupted treatment for 3–2–3–2–3 days, 0·1 per cent, Sodium Sulphadimidine. Ten mixed cockerels and pullets per group.

FIG. 6



Interrupted treatment for 3–2–3–2–3 days, 0·032 per cent. Sodium Sulphaquinoxaline. Ten pullets per group.

There was a sixty per cent mortality in the untreated control group (Fig. 4), deaths being spread over a seven-day period, but no deaths were recorded in groups B to K (Fig. 4) which were treated with 0·15 per cent sodium sulphadimidine. In the untreated control group (Fig. 5) there was a seventy per cent mortality. When treatment with 0·1 per cent sodium sulphadimidine commenced at the ninety-sixth hour after infection (group B) a fifty per cent mortality rate was recorded. Thereafter, the infection was controlled in groups C to H, but there was some evidence of continued schizogony in groups J and K (Fig. 5) where one death occurred in each group. Sodium sulphaquinoxaline at the reduced concentration of 0·032 per cent effectively prevented any re-appearance of coccidiosis after the initial loss had occurred in group B (Fig. 6).

Effects of Nitrofurazone and Nicarbazin on the Development of E. necatrix

To observe the effects on the development of *E. necatrix* of the concentrations of Nitrofurazone and Nicarbazin usually recommended for the prevention of coccidiosis (Horton-Smith and Long, 1952; Barber, 1955; Cuckler and Malanga, 1955) the drugs were mixed in the dry mash at concentrations of 0·01 per cent and 0·0125 per cent respectively. Each drug was then fed to a group of twenty three-weeks old chicks from the twenty-fourth hour before infection with 50,000 sporulated oocysts until the end of the sixth day after infection. Two similar groups were treated from the forty-eighth hour after infection. The results are shown in Table 1.

Good control of the infection was obtained with 0·0125 per cent Nicarbazin, no deaths being recorded in group A which was treated from the twenty-fourth hour before infection. There were only four deaths in group B where treatment commenced at the forty-eighth hour after infection. In groups C and D, which received 0·01 per cent Nitrofurazone, a greater mortality was recorded over days 5 and 6 and in addition several deaths occurred during the two days following the end of treatment. The observation was repeated (Table 2), but on this occasion groups of thirty seven-weeks old cockerels were infected with 100,000 sporulated oocysts of *E. necatrix* and treatment was started in groups A and C forty-eight hours before infection. All groups were treated until the end of the tenth day after infection. No deaths were recorded in group A which received 0·0125 per cent Nicarbazin from the forty-eighth hour before infection, but a high rate of mortality was shown in all other groups.

Prevention of Weight Loss during Treatment with Nicarbazin

The degree of protection afforded by Nicarbazin in preventing the weight loss which accompanies intestinal coccidiosis is shown in Fig. 7. Forty six-weeks old cockerels on a diet containing 0·0125 per cent Nicarbazin were infected with 100,000 sporulated oocysts of *E. necatrix;* ten similar birds were infected but were not treated. The rates of mortality and the weight gains recorded over an eighteen

TABLE 1

EFFECT OF LOW CONCENTRATIONS OF NICARBAZIN AND NITROFURAZONE ON THE DEVELOPMENT OF *E. NECATRIX*

Three-weeks old chicks infected with 50,000 sporulated oocysts.

	Days after day of inoculation (=Day 0)											*Total mortality per cent.*
	0 — 1	*Day 0*	*0 + 1*	*0 + 2*	*0 + 3*	*0 + 4*	*0 + 5*	*0 + 6*	*0 + 7*	*0 + 8*	*0 + 9*	*0 + 10*	
							Deaths from E. necatrix *infection*						
Group A 20 chicks	0·0125 per cent Nicarbazin fed continuously in the dry mash from 0 — 1 to 0 + 6						—	—	—	—	—	—	0
Group B 20				0·0125 per cent Nicarbazin continuously in the dry mash from 0 + 2 to 0 + 6			4	—	—	—	—	—	20
Group C 20	0·01 per cent Nitrofurazone fed continuously in the dry mash from 0 — 1 to 0 + 6						6	2	3	1	—	—	60
Group D 20				0·01 per cent Nitrofurazone continuously in the dry mash from 0 + 2 to 0 + 6			2	3	2	4	—	—	55
Control Group 14	No treatment						1	8	2	—	—	—	79

TABLE 2

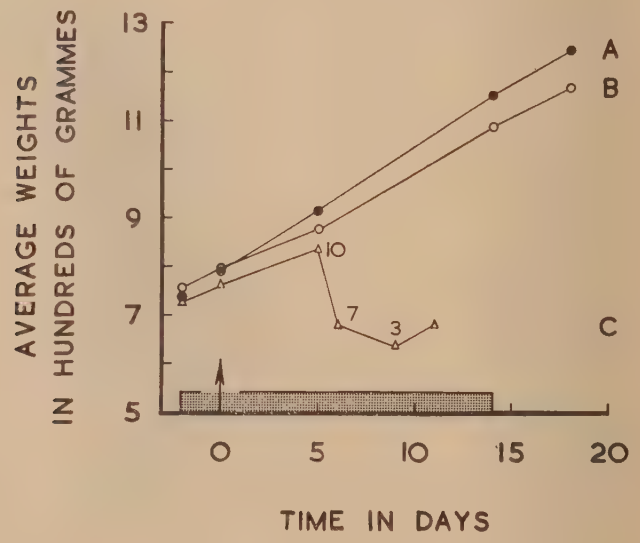
EFFECT OF LOW CONCENTRATIONS OF NICARBAZIN AND NITROFURAZONE ON THE DEVELOPMENT OF *E. NECATRIX*

Seven-weeks old birds infected with 150,000 sporulated oocysts.

	Days after day of inoculation (=Day 0)												*Total mortality per cent.*
	Day 0 — 2	0 — 1	0	0 + 1	0 + 2	0 + 3	0 + 4	0 + 5	0 + 6	0 + 7	0 + 8	0 + 9	0 + 10	
	Deaths from E. necatrix *infection*													
Group A 30 birds	0·0125 per cent Nicarbazin fed continuously in the dry mash from 0 — 2 to 0 + 10													
								—	—	—	—	—	—	0
Group B 30	0·0125 per cent Nicarbazin in the dry mash from 0 + 2 to 0 + 10													
								3	3	2	2	2	4	53
Group C 30	0·01 per cent Nitrofurazone fed continuously in the dry mash from 0 — 2 to 0 + 10													
								8	7	4	2	2	0	77
Group D 30	0·01 Nitrofurazone in the dry mash from 0 + 2 to 0 + 10													
								14	7	2	4	0	1	93
Control Group 23	No treatment							10	4	3	3	1	0	91

day period were compared with those of a group of twenty birds which were in isolation (Group A). Seven out of ten birds died in the untreated group C (Fig. 7) but no deaths were noted in group B which was treated with 0·0125 per cent Nicarbazin although slightly reduced weight gains were observed.

Fig. 7



The effect of 0·0125 per cent. Nicarbazin on the weight gains of six-weeks old cockerels infected with *E. necatrix*.

Group A; untreated uninfected controls.
Group B; infected and treated with Nicarbazin.
Group C; infected controls.

The shaded area denotes the period of treatment. The arrow indicates the time of infection.

Development of Resistance to Re-infection

In the experiment shown in Fig. 1, all surviving birds which had been treated with 0·2 per cent sodium sulphadimidine for 3 – 2 – 3 days, were given a challenge inoculum of 200,000 sporulated oocysts of *E. necatrix* on the twenty-first day after initial infection. Similarly, birds shown in Figs. 2, 3 and 6 were challenged on the twenty-sixth day. Following these challenge infections there were only three deaths from intestinal coccidiosis in the previously treated birds, but numerous colonies of schizonts were seen in a majority of the remainder at post-mortem examination ten days after challenge.

Of the thirty birds in group A (Table 2) which had received 0·0125 per cent Nicarbazin, ten were killed for post-mortem examination on the eleventh day after infection and revealed scattered

colonies of second generation schizonts. The remaining twenty birds were retained for a further two weeks and were then challenged with 100,000 sporulated oocysts. There were no deaths from this challenge, but most of the birds showed lesions of active schizogony at post-mortem examination on the twelfth day after re-infection. There was a steady gain in weight among treated birds (Fig. 7).

DISCUSSION

The successful control of outbreaks of caecal and intestinal coccidiosis in chickens depends primarily on an accurate diagnosis followed by prompt treatment. To be fully effective, treatment must be based on a knowledge of the life cycle of the particular parasite involved. In the past, control measures for coccidiosis have been based largely on a knowledge of the caecal form of the disease which is usually seen in young birds and if untreated can cause a high rate of mortality. It is generally accepted that older birds are more usually involved in outbreaks of intestinal coccidiosis and although, in the majority of cases, the death rate is substantially lower, an important aspect is the reduction in weight and general unthriftiness of affected birds. In addition, some birds in treated flocks do not appear to benefit from treatment and show active intestinal lesions for some time after treatment has ended. In the experiments described, the groups of birds may be regarded as units of a flock in which an outbreak of intestinal coccidiosis has been diagnosed and treatment has commenced on the day when symptoms first became apparent.

It is clear that the 3 – 2 - 3 type of interrupted treatment, which was devised for use against *E. tenella* (Davies and Kendall, 1954a, 1954b) was only partially effective in the control of intestinal coccidiosis caused by *E. necatrix*, for several deaths occurred after the end of treatment. It has been noted previously (Tyzzer, Theiler and Jones, 1932) that the schizogony of *E. necatrix* is more prolonged than that of *E. tenella*, i.e. the disease is not so precisely self-limiting, and it appeared that the second generation schizonts of the parasites were not controlled in those groups which in fact benefitted from only one three-day period of treatment, i.e., the second. A third period of treatment given after a further interval of two days effectively controlled this continued schizogony. Past experience has shown, however, that there is an increased danger of toxicity due to the sulphonamides if treatment at the approved concentrations is extended beyond the normally recommended six days. It will be noted that the rate of mortality from intestinal coccidiosis in certain of the control groups was higher than that which is usually experienced in outbreaks in the field. When the severity of the experimental disease was reduced, either by reducing the numbers of oocysts in the infecting dose, or by establishing infection in older birds, good results were obtained with lower concentrations of the sulphonamides.

It is probable, therefore, that better control of intestinal coccidiosis in the field will follow the use of concentrations of the sulphonamides lower than those which are recommended for the control of *E. tenella,* but that such concentrations should be administered intermittently over a thirteen day period. The low rate of mortality as a result of the challenge inocula indicates that following the interrupted treatments most of the birds had developed a substantial degree of resistance to re-infection. That this was not an absolute immunity to re-infection was proved by the renewed schizogony which was observed at post mortem examination ten days later.

In the prevention of intestinal coccidiosis caused by *E. necatrix* significantly better results were obtained with Nicarbazin than with Nitrofurazone. At 0·0125 per cent of the dry mash Nicarbazin prevented mortality provided birds were receiving the medicated ration before infection took place. In addition, during the treatment period a steady weight gain was observed among treated birds although there was some reduction in their growth rates when these were compared with normal controls. This reduction may possibly have been due to the effects of an invasion by sporozoites and the subsequent (partially inhibited) development of reduced numbers of second generation schizonts, for the results of the challenge infection showed that the birds had developed during treatment a substantial resistance to re-infection.

CONCLUSIONS

The 3 – 2 – 3 type of interrupted treatment with the sulphonamides, sulphadimidine or sulphaquinoxaline, was partially effective in the control of experimental intestinal coccidiosis caused by *E. necatrix,* but better results were obtained when an additional three-day period of treatment was given after a further interval of two days. When an infection of reduced severity was established in experimental birds good control was obtained with concentrations of the sulphonamides lower than those which are recommended for the control of *E. tenella.* It is suggested that better control of intestinal coccidiosis in the field may follow the use of these lower concentrations for the extended 3 – 2 – 3 – 2 – 3 schedule of interrupted treatment.

Nicarbazin at 0·0125 per cent was more effective than Nitrofurazone at 0·01 per cent in the prevention of intestinal coccidiosis caused by *E. necatrix.* Provided treatment was started before infection took place, Nicarbazin prevented the appearance of symptoms, allowed a steady weight gain during treatment and did not prevent the development of a substantial degree of resistance in treated birds.

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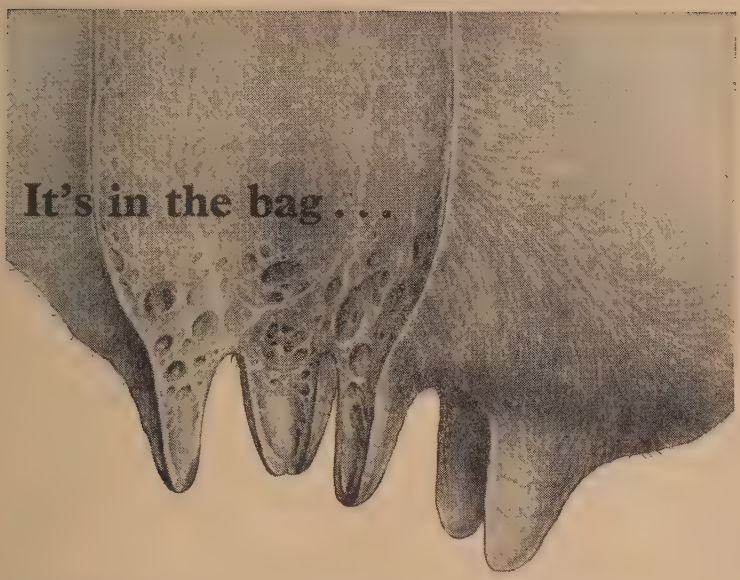

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